UNIVERSITY OF ALBERTA

REDUCTION OF CHLOROPHYLL ACCUMULATION IN SEED OF TRANSGENIC *BRASSICA NAPUS* USING ANTISENSE TECHNOLOGY

By

JANICE CAROLE POLITESKI MORISSETTE ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science.

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DEPARTMENT OF PLANT SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Reduction of Chlorophyll Accumulation in Seed of Transgenic *Brassica napus* using Antisense Technology** submitted by **Janice Carole Politeski Morissette** in partial fulfillment of the requirements for the degree of **Master of Science** in **Plant Molecular Biology and Biotechnology.**

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"Knowledge is power."

- anonymous

DEDICATION

This thesis is lovingly dedicated to my husband, Pierre.

ABSTRACT

As little as 6% green seed in a canola crop can significantly reduce the economic value of a harvest. The chlorophylls and pigments remaining in green seed cause the extracted oil to retain a green color which must be removed through procedures that reduce the overall oil yield. Thus, reducing the amount of pigment in the seed is an industry priority. In an effort to reduce the amount of chlorophyll in mature seed, canola (*Brassica napus* cv. Westar) cotyledons have been transformed using *Agrobacterium tumefaciens* containing an antisense construct of a type I cab gene of Photosystem II. A napin promoter has been used to target antisense cab expression to the seed. Two transgenic plant lines were obtained and characterized. Results of chlorophyll analyses indicate that under greenhouse conditions, chlorophyll levels are significantly reduced in transgenic seed compared to Westar seed. Western blot analyses of Cab proteins suggest that accumulation of Cab proteins in transgenic seed is also reduced compared to Cab protein levels in Westar seed. Polymerase Chain Reaction (PCR) and Southern blot analysis of the transgenic lines indicate stable inheritance of the antisense cab construct through two successive generations. The results of this study support the use of antisense cab as an effective means of reducing chlorophyll accumulation in the seed of transgenic canola. The results also support the idea of a codependent relationship between chlorophyll and Cab protein accumulation. A number of experiments remain to be performed in order to determine the effect of the antisense cab gene on seed storage protein and lipid accumulation.

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LIST OF ABBREVIATIONS

35S	promoter region of the cauliflower mosaic virus
A	absorbance
ABA	abscisic acid
ALA	5-aminolevulinic acid
AMV	avian mosaic virus
ATP	adenosine triphosphate
BA	benzyladenine
BCA	bicinchoninic acid
BSA	bovine serum albumin
bp	base pair
cab	gene encoding chlorophyll a/b binding protein
Cab	chlorophyll a/b binding protein
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
chl	chlorophyll
cm	centimeter
cv	cultivated variety
DAP	days after pollination
dd	double distilled
DNA	deoxyribonucleic acid
dNTP	dideoxyribonucleotide
DPA	days post anthesis
E	Einstein
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
FDA	food and drug administration

Gus	β-glucuronidase (gene)
Kb	kilobase
Kd	kilodalton
LB	Luria Bertani media
LEA	late embryogenesis abundant
LHC	light harvesting complex
m	meter
mA	millampere
mas	mannopine synthase gene
mg	milligram
mm	millimeter
mM	millimolar
M	molar
mRNA	messenger ribonucleic acid
MS	Murashige Skoog medium
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
nos	nopaline synthase gene
nptII	neomycin phosphotransferase II (gene)
OD	optical density
ori	origin of replication
^{32}P	radioactive phosphorus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfr	phytochrome (active form)
ppm	parts per million

Pr	phytochrome (inactive form)
PS	photosystem
rpm	revolutions per minute
s	second
SAS	statistical analysis software
SDS	sodium dodecyl sulfate
SE	standard error
TAE	Tris-acetic acid-EDTA
TCA	trichloroacetic acid
T-DNA	transfer deoxyribonucleic acid
TE	TRIS EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamene
Ti	tumour inducing
TRIS	tris(hydroxymethyl)methylamine
tRNA	transfer ribonucleic acid
v/v	percent “volume in volume”
V	volt
VLFR	very low fluence response
vir	virulence region of Ti plasmid
w/v	percent “weight in volume”
x-gal	5-bromo, 4-chloro, 3-indolyl β-D-galactoside
x-gluc	5-bromo, 4-chloro, 3-indolyl glucuronide
µg	microgram
µl	microlitre

1: INTRODUCTION

Canola has become the most important oilseed crop in Canada. Canadian canola oil and meal earned 322 million dollars in export sales in 1992 (International Markets Bureau, 1994). The harvesting and production of canola and canola products has become a multimillion dollar Canadian industry.

One of the limitations of canola as a crop is its tendency to produce green seed after exposure to a variety of environmental stresses, including cold weather. Exposure to sub-lethal frost induces renewed synthesis of chlorophyll and chlorophyll a/b binding (Cab) proteins in canola seed resulting in mature seed containing significant amounts of pigment (Johnson-Flanagan et al., 1990, Green et al., 1995). During the crushing process, both pigments and lipids are extracted from the seed, resulting in tainted oil. Industrial processing is required to remove the contaminating pigments from the extracted oil. This is expensive and results in reduction in overall oil yield.

The use of bleaching clay has been a fairly effective but expensive method of pigment removal from canola oil. It may be possible to circumvent the need for pigment removal by developing canola lines that do not readily lend themselves to green seed production. This study has attempted to develop such a canola line based on *Brassica napus* cv. Westar using *Agrobacterium tumefaciens*-mediated transformation and antisense technology. It was the intent of this study to take advantage of the hypothesized codependent relationship between chlorophyll and Cab proteins (Plumley and Schmidt, 1995) by reducing Cab protein, and consequently lowering chlorophyll accumulation in the seed.

FIG. 1: A canola field at the University of Alberta farm in Edmonton, Alberta.

2: LITERATURE REVIEW

2.I: CANADA'S CANOLA INDUSTRY

2.I.a: Production of canola in Canada

Canola is one of Canada's largest agricultural export products. In 1990, canola was second only to wheat as revenue generator among crops grown in Canada and is currently considered to be the most important oilseed crop in Canada (Canola Council of Canada, 1991). In 1992, 1.79 million tonnes of Canadian canola seed was exported to various countries. This figure has increased to 2.98 million tonnes exported in 1993/94 (International Markets Bureau, 1994, DeClerq et al., 1994). The export of Canadian canola oil and meal earned $322 million dollars in 1992, the major export market being the United States. Total domestic and export canola oil and meal sales in 1992 amounted to $921 million dollars (International Markets Bureau, 1994). In 1995, 5.3 million hectares of canola were planted and 6.37 million tonnes of canola were harvested in Canada, with Alberta making up 39% of 1995 canola production (DeClerq et al., 1995).

Canola was developed by Dr. Baldar Stefansson in 1974 when he produced the first "double-low" variety of rapeseed, which contained reduced erucic acid (less than 5%) in the oil and 3 mg/g or less of glucosinolate in the meal. In 1986 the Tradesmarks Branch of Consumer and Corporate Affairs decided that canola oil must contain less than 2% erucic acid and less than 30 µmol/g glucosinolates in the meal (Canola Council of Canada, 1991).

The Canadian Grain Commission sets and maintains strict grading standards in order to determine the quality of canola harvests year after year. There are three grades of canola based in part on percentage of green seed within a crop. Canola No. 1 is superior grade canola which may not contain over 2% green seed. Canola No. 2 is of a lesser grade and may not contain over 6% green seed. Canola No. 3 is a fairly low grade canola which must not contain over 20% green seed (Canola Council of Canada, 1991). In Sweden, seed prices have also been regulated, in part, according to the occurrence of green seed in a canola crop. The prices were calculated according to chlorophyll content in oil extracted from seed samples. In 1973, the maximum price reduction was 56% of the base seed price for crops containing 110 ppm chlorophyll in extracted oil (Dahlen, 1973). Thus, for many years it has been of benefit to the canola producer to grow canola crops with very low occurrence of green seed. High quality canola crops with low occurrence of green seed bring great economic benefits to the producers because they require less processing to remove contaminating pigments.

2.I.b: Methods of oil extraction and pigment removal

Canola oil has a wide number of uses including salad dressing, shortenings, margarine, printing inks and industrial lubricants. In 1989, canola oil made up 63% of all vegetable oils and 82% of all salad oils produced in Canada (International Markets Bureau, 1994).

The first step in processing raw canola seed into a usable oil product is extraction of the oil from the seed. Canola oil extraction begins with the flaking of the seed into very thin pieces in order to achieve a high surface to volume ratio. The flakes are then cooked in order to coalesce the small droplets of oil by reducing oil viscosity. Cooking occurs at 77-100° C with a holding time of 15-20 minutes to facilitate good oil release. The cooked and flaked canola is then pressed gently to reduce oil content from 42 to 14% in the resulting meal. The meal is packed into larger fragments that are solvent extracted with hexane to remove most of the remaining oil. The solvent-extracted oil is removed from the hexane and then combined with the expressed oil to make up the crude oil fraction.

The crude oil is degummed by hydrating with steam to remove phosphatides and free fatty acids. The degummed crude oil then undergoes refining in order to remove most of the free fatty acids, phospholipids, chlorophylls and other color bodies, iron and copper from the oil.

In Canada, the main method of purifying canola oil is alkali refining. The crude oil is treated with phosphoric acid to condition the phospholipids making them less soluble in the oil and more easily removed. Following phosphoric acid treatment, a sodium hydroxide solution is added to the oil and the resultant mixture is agitated. The oil is then washed several times with water to lower the soap content to below 50 ppm. The refined oil is then dried to remove water. The refined oil can then be hydrogenated or interesterified and blended with other oils to form margarine, shortening, or a variety of other products.

In order to remove odor and flavor compounds to produce a desirable bland oil, the oil must be deodorized. The oil is steam distilled under vacuum at high temperature. The highly volatile odor and flavor compounds are preferentially removed during steam distillation. After the deodorized oil is cooled, 0.005-0.01% citric acid is added to prevent oxidation.

While the process of canola oil extraction is fairly complex, it is nonetheless quite efficient. Virtually all of the oil from the canola seed can be extracted during the primary processing. Of the 42% oil content found in canola seed, 28% can be extracted from the pre-pressing process and 14% can be obtained from the solvent extraction process (Vaisey-Genser and Eskin, 1982).

2.I.c: Effect of green seed on processing of canola oil products

It is during the primary processing of canola seed that the presence of green seed becomes evident. When seed containing an excessive amount of chlorophyll are crushed, a great deal of the chlorophyll in the seed is extracted with the oil thus tainting the oil with a green color (Singh and Chuaqui, 1991). Pigment content is, in fact, one measure of canola oil quality. Chlorophyll that is extracted into the crude oil negatively affects the oil in two ways; by reducing oil hydrogenation, and by increasing oil oxidation.

Hydrogenation modifies the physical properties of the oil as well as rendering it less susceptible to damage caused by oxidation and heat. Hydrogenation is caused by the addition of hydrogen at the double bonds of unsaturated fatty acids under conditions of high temperature and pressure in the presence of an appropriate catalyst such as nickel. The double bonds of the triglyceride become more saturated, thus changing the fatty acid composition of the oil. The increase in saturation results in a physically harder oil product which is more resistant to oxidation. This process of hydrogenation is used for the production of margarine and shortening (Vaisey-Genser and Eskin, 1982).

The presence of chlorophyll in oil appears to negatively influence the rate of hydrogenation. Studies conducted by Abraham and deMan in 1986 examined the effect of chlorophyll on hydrogenation of canola oil. Different amounts of chlorophyll a were added to canola oil samples prior to hydrogenation. The hydrogenated oil samples were then examined for solid fat content and fatty acid composition. Following hydrogenation it was found that oil samples with higher amounts of chlorophyll contained lower solid fat content and higher levels of unsaturated fatty acids compared to oil samples containing no chlorophyll. It has been suggested that chlorophyll physically blocks the active centers of the catalyst thus inhibiting hydrogenation (Abraham and deMan, 1986).

Chlorophyll has also been found to increase the rate of oil oxidation causing rancidity in the refined oil. The oxidation rate of oil is determined by a number of factors including the presence of O_2, light, temperature cycling, degree of unsaturation of the fatty acids, antioxidants, and prooxidants, including metals and chlorophyll.

The mechanism of oxidation involves the abstraction of a hydrogen ion adjacent to a fatty acid double bond by light, heat and a metal to form a free radical. The free radical then combines with oxygen to form a peroxy radical which abstracts a hydrogen ion from another unsaturated fatty acid to form a hydroperoxide and another free radical. This reaction perpetuates itself in a type of chain reaction.

The hydroperoxides decompose to form a series of secondary oxidation products such as aldehydes, ketones, and alcohols, which have disagreeable flavors and odors (Vaisey-Gense and Eskin, 1982). Experiments conducted in Sweden in the late 1960's examined the effects of chlorophyll content on storage stability of refined oils. Taste tests were conducted and peroxide levels were examined for bleached, refined oils derived from crude oil with varying chlorophyll contents. Taste tests revealed that oil derived from crude oil containing less than 20 ppm chlorophyll was deemed to be of better quality compared to oils containing 20-40 ppm chlorophyll. Furthermore, peroxide values within refined oils derived from high chlorophyll content crude oils were found to increase substantially over a period of several weeks storage compared to bleached oils refined from low chlorophyll crude oil (Dahlen, 1973). The results of these studies indicate that chlorophyll is capable of accelerating the oxidative process and thereby reduces storage stability of the oil (Dahlen, 1973, Usuki et al., 1984).

2.I.d: Removal of pigments from extracted oil

In order to minimize the negative effects that chlorophyll has on oil processing, a number of methods have been developed to remove chlorophyll from crude oil. Once the pigments are removed, the crude oil can be further refined and prepared for the marketplace.

Bleaching is currently the most popular means of removing chlorophyll pigments from extracted oil. In Canada, canola oil is bleached by agitating it under vacuum with 0.125-2.0% bleaching clay. This bleaching process also removes products of oxidation, residual soap and other products that could negatively affect the hydrogenation process. The chlorophyll content of unbleached refined oil is reduced from 12.8 ppm to 0.2 ppm in the presence of 1% clay, while no chlorophyll can be detected in the presence of 3% clay (Vaisey-Genser and Eskin, 1982). Bleaching is quite expensive in terms of the cost of the clay and the bleaching clay itself absorbs a great deal of oil (as much as ¾ the weight of the clay). Nevertheless bleaching remains the most popular method of pigment removal (Diosady, 1991).

A recently explored method of chlorophyll removal involves the use of ion exchange resins during the various stages of oil processing. One limitation of this chlorophyll removal technique involves competition between the chlorophyll and free fatty acids for ion exchange sites in the resin. This competition between fatty acids and chlorophyll reduces the efficiency with which chlorophyll is removed from the oil. A maximum of 90% chlorophyll removal was seen for short periods of time in tests conducted by Diosady (1991). The residual chlorophyll concentration eventually rises to about 80% of the chlorophyll concentration in the feed. The cost of the resin is even more expensive than the cost of chlorophyll removal by bleaching and is impractical for use on an industrial scale (Diosady, 1991).

Tests conducted on oil refined from high chlorophyll crude oil revealed that, even after bleaching, storage stability of the refined oil was significantly lower compared to oil refined from low chlorophyll crude oil (Dahlen, 1973). This indicates that the negative effects of high chlorophyll levels can still be observed in the refined oil.

The removal of chlorophyll during the extraction process seems to be an economically inefficient means of keeping chlorophyll out of refined oil. Both bleaching and ion exchange resins are very expensive methods of chlorophyll removal. In addition, bleaching removes both oil and chlorophyll, thereby reducing the oil yield and adding to the expense of processing oil. A biomolecular approach to the reduction of chlorophyll accumulation in seed may prove to be a more cost effective means of controlling oil chlorophyll content than expensive oil processing methods.

In order to design a method to reduce endogenous chlorophyll levels, one must first understand how the seed itself develops and matures and how chlorophyll and other photosynthetic compounds are produced and organized within the developing seed. Once the fundamentals of seed greening and degreening are understood, one can then attempt to understand how normal degreening can be arrested and result in green seed. With an understanding of the processes that may interrupt degreening, such as sub-lethal frost, one can then devise a method to circumvent those processes and reestablish effective seed degreening.

2.II: GREENING OF CANOLA SEED

2.II.a: Chlorophyll biosynthesis

As a canola seed develops from a fertilized ovule into a mature, germinable structure, it goes through a vast number of changes. These include increases in size and weight as the embryo and endosperm develop, and production and storage of oil bodies and seed storage proteins during seed development. Many of these changes manifest themselves in ways that can be seen or measured in the mature seed, such as increase in seed size or accumulation of a certain storage protein. However, there are also a number of changes that occur during seed development that are transitory. This refers to changes that occur in the seed for which there is little or no direct evidence in the mature seed. Such changes include changes in moisture content during seed development. For instance, at 15 days post anthesis (DPA) seed moisture content is around 80% , while in a mature seed, the seed moisture content falls to 4-11%. Other transitory changes seen during seed development are the accumulation and subsequent degradation of chlorophyll and associated photosynthetic products. Canola seed accumulates chlorophyll during seed development, with maximum accumulation around 28 DPA, then chlorophyll degrades (Johnson-Flanagan, unpublished). This process of chlorophyll accumulation and degradation during seed development is referred to as seed greening and degreening.

Seed greening is the process whereby developing seed accumulates chlorophyll and related photosynthetic components resulting in the development of immature green seed. Chlorophyll accumulation in seed is expected to follow the same principles as chlorophyll accumulation in other photosynthetic tissues, including the same synthetic pathways, chlorophyll species, and modes of regulation.

Chlorophyll production is found within photosynthetic plant tissues such as leaf and stem. Within the plant cell itself, chlorophyll production occurs within organelles called chloroplasts which develop from progenitor organelles called proplastids. Proplastids are the progenitors of many plastid types including chloroplasts. They are found in root and shoot meristems, embryos, endosperm and young leaves. Proplastids contain very few thylakoid membranes. As development occurs the proplastid accumulates starch and is referred to as an amyloplast. The starch deposits are slowly lost and the plastid grows in size and develops stroma lamellae. Upon maturation the stromal thylakoids align, the disc-shaped thylakoids of grana become stacked and the photosynthetic components are laid down to result in a mature chloroplast (Dennis and Turpin, 1990).

There is a distinct anatomical organization of membranes within the mature chloroplast and the components of the photosynthetic apparatus are themselves organized within these membranes. The plastid is surrounded by a membrane envelope comprised of both an inner and outer membrane separated by 10-20 nm. This intramembranous space is one of three compartments within the chloroplast. There is also an intricate internal membrane system made up of interconnected membrane sacs called the thylakoid. The thylakoid is located in the internal space of the chloroplast referred to as the stroma, the second chloroplastic compartment. The thylakoid membrane system is divided into two types of structures: granal and stromal thylakoids. Stacks of flattened disc-shaped thylakoid membrane sacs are referred to as grana. There are typically 50 grana per angiosperm chloroplast. The degree of stacking depends on the physiological requirements of the plant. These granal stacks are connected by membrane channels that traverse the stroma and are called stromal lamellae or stromal thylakoids. The spaces within the thylakoid membrane system make up the third compartment found within the chloroplast (Dennis and Turpin, 1990). It is within the stromal region of the chloroplast that chlorophyll is produced.

Chlorophyll is a complex molecule consisting of a tetrapyrrole ring with a magnesium atom at its center (von Wettstein et al., 1995). It is produced exclusively in the chloroplast through a series of reactions including the C5 pathway. The C5 pathway is a three step pathway which converts glutamate into 5-aminolevulinic acid (ALA), a chlorophyll precursor. Glutamic acid is converted to its active form by ligation to tRNA^{Glu} with the help of the aminoacyl tRNA synthetase in the presence of ATP and Mg^{2+}. The resulting glutamyl tRNA^{Glu} is reduced by a reductase in an NADPH-dependent reaction to glutamate-1-semialdehyde. An aminotransferase then converts glutamate-1-semialdehyde into ALA (Kannangara et al., 1994).

In the chloroplast, two molecules of ALA are converted into one porphobilinogen molecule through the action of a dehydratase enzyme and the release of one water molecule. Four molecules of porphobilinogen are deaminated in a stepwise fashion through the action of porphobilinogen deaminase to form the tetrapyrrole hydroxymethylane. The tetrapyrrole is then closed into a ring by uroporphyrinogen III synthase. The closed tetrapyrrole ring (uroporphyrinogen III) is then decarboxylated and oxidized twice to form protoporphyrin IX, which is red in color (von Wettstein et al., 1995).

Magnesium is added to the center of the tetrapyrrole ring through the action of a Mg-chelatase. The resulting Mg-protoporphyrin IX undergoes a methyl transferase reaction to form the monomethyl ester. A cyclase reaction results in divinyl protochlorophyllide. Divinyl protochlorophyllide is reduced to form protochlorophyllide, which, in the presence of NADPH, water and light, is reduced to chlorophyllide a and phytyl pyrophosphate. A final esterification of the propionic acid side chain of chlorophyllide a by chlorophyll synthetase results in production of one molecule of chlorophyll a (Rudiger et al., 1980, von Wettstein et al., 1995).

There are two main species of chlorophyll evident in higher plants. The most abundant chlorophyll species is chlorophyll a which is found in both Photosystem I and II. Chlorophyll a contains a methyl group at position three of the chlorophyll molecule.

Chlorophyll b is a derivative of chlorophyll a formed by replacing the methyl group at position three of the chlorophyll molecule with a formyl group. The formyl group is inserted into the chlorophyll b molecule through the action of an oxygenase enzyme. The oxygenase enzyme incorporates atmospheric oxygen to create the formyl group found in chlorophyll b (Porra et al., 1994). Chlorophyll b is less abundant than chlorophyll a.

Despite the fact that chlorophyll is synthesized within the chloroplast and functions only within the chloroplast, the enzymes responsible for the production of chlorophyll are all nuclear encoded. All genes encoding C5 pathway enzymes, as well as genes encoding enzymes responsible for the conversion of ALA into chlorophyll a , are transcribed in the nucleus. Transcripts are translated on cytoplasmic ribosomes and transported into the chloroplast via transit peptides attached to the N-terminal end of the peptides.

2.II.b: Regulation of chlorophyll synthesis

In the past ten years, a number of systems have been suggested as methods of regulating chlorophyll synthesis, including a system of negative feedback to regulate expression of the reductase encoding gene, hemA (Tanaka et al., 1996). Recently it has become clear, however, that light remains the primary means of regulating chlorophyll production.

A number of steps leading to chlorophyll synthesis require the presence of ATP and/or NADPH. The conversion of glutamyl $tRNA^{Glu}$ to glutamate-1-semialdehyde in the C5 pathway requires NADPH. The conversion of protoporphyrin IX to Mg-protoporphyrin requires ATP. In addition, the conversion of protochlorophyllide to chlorophyllide a requires NADPH as well as light. ATP and NADPH are high energy molecules produced during the photosynthetic process. Although ATP can be produced by other means, NADPH is generated solely by photosynthesis. Since photosynthesis cannot occur in the absence of light, those biosynthetic steps which require the presence of ATP and particularly NADPH are indirectly regulated by light (Bhaya and Castelfranco, 1985).

Light plays a direct role in the conversion of protochlorophyllide to chlorophyllide a. This conversion requires NADPH. The enzyme responsible for the conversion, protochlorophyllide oxidoreductase, is a light dependent enzyme. Dark germinated barley seedlings have been found to accumulate protochlorophyllide but will not produce chlorophyll. However, upon exposure to light, the protochlorophyllide is elevated to an excited state and the oxidoreductase is activated and reduces the protochlorophyllide to chlorophyllide a (Henningsen et al., 1993). Once this occurs chlorophyll synthetase becomes activated and catalyses the conversion to chlorophyll a (Lindsten et al., 1993). Light therefore, plays a major regulatory role in the conversion of protochlorophyllide to chlorophyll a.

Light appears to have additional regulatory roles. In chlorophyll biosynthesis, it is thought that the formation of ALA is limiting because the rate of ALA formation seems to parallel that of chlorophyll accumulation (Beale and Weinstein, 1990). Recently, experiments conducted by Tanaka et al. (1996) have revealed that there are two genes (hemA1 and hemA2) encoding the glutamyl-tRNA reductase enzyme in the C5 pathway. It is believed that the hemA1 gene is light inducible and allows for the production of ALA that can subsequently be used for chlorophyll synthesis. The hemA2 gene is believed to be responsible for the production of porphyrins required for the production of other tetrapyrroles such as heme. The hemA2 gene is not light inducible. It has been suggested that excesses of ALA made by the product of the light inducible hemA1 gene might repress the hemA2 gene expression in hypocotyls. In this way the plant may be focusing its energies on the production of chlorophyll in the presence of light. It appears that light can directly upregulate expression of hemA1 glutamyl-tRNA reductase, thereby allowing increased production of ALA and, consequently, increased chlorophyll production (Tanaka et al., 1996). Light also plays an important part in regulation of chlorophyll synthesis through production of the photosynthetic molecules ATP and NADPH. All chlorophyll molecules, once synthesized, are noncovalently bound to proteins in the granal and stromal thylakoid membranes within the chloroplast (Jansson, 1994). The proteins to which the chlorophyll molecules bind are referred to as chlorophyll binding proteins.

2.II.c: Chlorophyll binding protein biosynthesis

The term chlorophyll binding protein refers to all proteins found within the thylakoid membrane of the chloroplast that bind chlorophyll and other pigments to form chlorophyll/protein complexes. There are a variety of chlorophyll binding proteins, a few of which bind only chlorophyll a. However, the most abundant chlorophyll binding proteins are the light harvesting chlorophyll a/b binding (Cab) proteins that bind chlorophyll a, chlorophyll b, and some carotenoids. The expression of the various chlorophyll binding proteins must be coordinated between the chloroplast and the cell nucleus in order to ensure that photosynthesis occurs effectively.

The chloroplast genome contains several of the genes encoding chlorophyll binding proteins (Mullet, J., 1990). The psbA and psbD genes encode proteins that bind reaction center chlorophyll a molecules, while psbB and psbC genes encode proteins that serve a light harvesting function but bind only chlorophyll a (Baumgartner et al., 1993, Holschuh et al., 1984, Kim and Mullet, 1995, Westhoff and Herrmann, 1988). Though important to the function of the photosynthetic process, these proteins are not as abundant and do not bind as much chlorophyll as some of the nuclear encoded proteins.

The plant cell nucleus also contains many genes encoding chlorophyll binding proteins. Most of these proteins encoded by the nuclear genes can bind both chlorophyll a and chlorophyll b and thus are referred to as chlorophyll a/b binding (Cab) proteins. The cab genes are divided into a series of small multigene families based on sequence homology (Dunsmuir, 1985). The number of gene subfamilies and the number of genes within each subfamily is species specific. In petunia, there are 16 cab genes divided into 5 subfamilies, while in tomato, 12 subfamilies of cab genes have been identified (Dunsmuir, 1985, Piechulla et al., 1991).

Since Cab proteins must be transported across the chloroplast membrane, a transit sequence at the N-terminal end of the polypeptide is necessary. The coding regions for the transit sequence of the different cab genes seem to have a greater nucleotide divergence than do the protein coding regions. There is generally 75% homology at the nucleotide level and 85% homology at the amino acid level in the protein coding regions (Stayton et al., 1986). Though all cab genes share up to 75% sequence homology, within a single cab subfamily, sequence homology can exceed 75% (Green, 1988).

Each of the cab subfamilies can be divided into one of three classes, type I, II, or III. Type I genes lack any introns (White et al., 1992). Type II genes contain one intron (Falconet et al., 1991), and type III genes contain two introns (Falconet et al., 1993).

Nuclear encoded cab genes are transcribed in the nucleus by RNA polymerase II. The resulting mRNA is then spliced to remove introns, capped on the 5' end with 7-methylguanosine residues, and polyadenylated on the 3' end to produce a mature mRNA (Lefebvre, D., 1990). The mRNA is then transported to the cytoplasm where it binds to and is translated by an 80S ribosome to produce the Cab apoprotein (approximately 32,000 MW). Following complete translation of the apoprotein, it is then transported in an energy dependent manner across the chloroplast envelope (Bennett, 1981). It remains uncertain at which precise point the N-terminal transit sequence is cleaved from the apoprotein, but cleavage is believed to occur soon after transport across the chloroplast membrane.

In an undetermined series of events, the apoprotein is incorporated into the thylakoid membrane and processed into a variety of chlorophyll/protein complexes. Until recently it was believed that the Cab apoprotein was incorporated into the thylakoid, processed into its mature form, and then assembled into chlorophyll/protein complexes. However, a recent study revealed type II apoprotein intermediates associated with the light harvesting complex (LHC) for Photosystem II (Sigrist and Staehelin, 1994). These results suggest that complete processing of type II apoproteins is not required for assembly and may be accomplished following their assembly into the LHCII complex. Though the Sigrist and Staehelin study does shed some light on the formation of chlorophyll/protein complexes, the complete series of events leading to assembly of mature chlorophyll/protein complexes has not yet been fully elucidated.

The transcription and translation of chloroplastic genes encoding chlorophyll a binding proteins begins with co-transcription of certain polycistronic plastid gene pairs such as psbD/psbC by chloroplast RNA polymerase. The resulting RNAs are not capped or polyadenylated but introns are removed. Transcription, processing of RNA, and translation all occur within the plastid compartment suggesting a coupling of transcription and translation. Translation occurs on 70S ribosomes. Some proteins can be inserted cotranslationally into the thylakoid membrane while other proteins, such as psbA, require C-terminal cleavage for assembly into chlorophyll/protein complexes (Dennis and Turpin, 1990).

2.II.d: Regulation of chlorophyll binding protein biosynthesis

Chloroplastic and nuclear genes encoding chlorophyll binding proteins are regulated in different ways. Accumulation of chloroplast encoded chlorophyll binding proteins appears to be regulated primarily during and following translation while nuclear encoded cab genes are also regulated during transcription.

2.II.d.1: Regulation of chloroplast encoded chlorophyll binding protein synthesis

Regulation of some chloroplastic genes involves a requirement for light. Chloroplastic chlorophyll binding protein genes such as psbB and psbC are transcribable in the dark. RNA transcripts for these genes are abundant and found on plastid polysomes in dark grown tissues. However, the proteins are not found in dark grown tissues. Protein accumulation in the dark may be blocked by polypeptide translation or rapid protein turnover. The light induced accumulation of psbA, B, and C seems to be mediated by protochlorophyllide. Protochlorophyllide needs to be excited by light energy in order to be reduced to chlorophyllide then chlorophyll. Chlorophyll is believed to bind to nascent chains in order to stimulate translation or stabilize apoproteins and allow these chlorophyll binding proteins to accumulate to high levels in the thylakoid membranes. Stimulation of translation by binding chlorophyll to nascent chains is referred to as regulation at the level of translation, whereas stabilization of apoproteins by binding of chlorophyll is known as post-translational regulation (Mullet, J., 1990).

2.II.d.2. Regulation of nuclear encoded Cab protein synthesis

The biosynthesis and accumulation of nuclear encoded Cab proteins is regulated at a variety of levels including transcriptional, post-transcriptional and post-translational levels (Apel, 1978, Bennett, 1981, Flores and Tobin, 1988). Each level of regulation is affected by a number of factors. In recent years, the study of cab gene expression has focused on the regulation of the genes encoding the apoproteins for the light harvesting complex of Photosystem II (LHCII). Since

LHCII apoproteins make up 50% of the protein in the thylakoid and bind half of all chlorophyll a and most chlorophyll b in plant cells (Bassi et al., 1990), they are of primary interest and will be the focus of this study of Cab protein regulation.

2.II.d.3: Transcriptional regulation of cab genes

At the level of transcriptional control, cab genes are affected by light, through phytochrome and very low fluence (VLF) induced circadian rhythms, by hormones, such as abscisic acid (ABA) and cytokinins, and by developmental cues. Studies conducted in 1978 by Klaus Apel showed that a dark grown barley plant contained negligible amounts of cab mRNA. However, exposure to a 15 second red light pulse induced accumulation of cab mRNA in the same dark grown plant. This red light effect could be countered by exposure to far red light. Such a series of occurrences is indicative of a phytochrome response. Red light converts the pigment Pr into the active Pfr which is responsible for inducing formation of cab mRNA. In the dark or with exposure to far red light, Pfr is converted back to the inactive Pr, which explains reduced induction of cab mRNA synthesis in the dark several hours after the red light pulse (Bennett, 1981).

Another form of transcriptional regulation involving light concerns the circadian expression of cab genes. Cab genes are regulated by an endogenous rhythm which induces changes in cab mRNA steady-state levels. The highest cab mRNA levels are seen about 5 hours after dark to light transitions, thus mRNA levels decrease in late afternoon and night (Kellmann et al., 1989). Under conditions of constant light and temperature, the diurnal rhythm has about a 32 hour period length. However, in conditions of alternating light and dark or alternating temperature, period length falls to 24 hours and the amplitude of cab mRNA increases several fold over constant conditions (Riesselman and Piechulla, 1990).

The results of recent studies suggest that the setting of the circadian clock is a very low fluence response (VLFR). A VLFR can be induced by as little as 0.003% active labile phytochrome (DePetter et al., 1988). In fact, far red light, which normally reverses phytochrome responses, converts 3% of Pr to Pfr, a saturating dose of Pfr to induce a VLFR. Moonlight or starlight can also induce VLFRs (Nagy et al., 1993). Studies conducted by White have shown that two type I cab genes are induced by VLF light. It is the VLF response as well as the phytochrome response that regulate the diurnal pattern of cab transcript accumulation (White et al., 1995).

Cab mRNA levels are also sensitive to the effects of several hormones, including abscic acid (ABA) and cytokinins. Experiments conducted with 35 DPA soybean embryos indicated that in cultured excised cotyledons, cab mRNA levels were very high while endogenous ABA levels were very low. However, in 70 DPA cotyledons, cab mRNA levels were very low while endogenous ABA levels were very high. The results of these experiments suggest that there is a strict correlation between high endogenous ABA and declining cab mRNA levels (Chang and Walling, 1991).

Cytokinins, another class of plant hormones, have also been found to influence cab mRNA accumulation. Normally there is very little cab mRNA in dark grown plants. However, studies have shown dark grown *Lemna gibba* plants treated with exogenous cytokinins, such as benzyladenine (BA), experience an increase in abundance of cab mRNA. A more detailed examination of the cab mRNA levels indicated that 24 hours after a BA treatment a 1.5 fold enhancement in cab transcription rates is detected (Flores and Tobin, 1988). The effect of BA treatment on dark grown *Lemna gibba* plants appears to not only be enhancement of transcription, but also a 4.8 fold increase in mRNA accumulation. It seems unlikely that a 1.5 fold increase in transcription rates could result in such a large accumulation of mRNA. It is, therefore, suggested that cytokinins also exert post-transcriptional controls to allow accumulation of cab mRNA (Flores and Tobin, 1988).

2.II.d.4: Post-transcriptional regulation of cab genes

Cab mRNA transcription is also regulated by developmental cues. Cab mRNA levels have been seen to fluctuate in embryos during seed development and maturation as well as in cotyledons during early seedling development (Mullet, 1988, Taylor, 1989). Particular stages of development, therefore, also seem to influence cab mRNA transcription.

There is an additional means of post-transcriptional regulation that affects the accumulation of Cab proteins. However, the mechanism of regulation is not yet understood. Experiments conducted by Flachmann and Kuhlbrandt in 1995 examined the effect of cab mRNA levels on Cab protein accumulation in the thylakoid. The cab mRNA levels within tobacco plants were manipulated and the resulting effect on Cab protein levels determined. It was revealed that although cab mRNA can be reduced to almost undetectable levels, the amount of Cab protein remained almost unchanged compared to control plants. These results suggest that there is a yet undiscovered post-transcriptional process that selects particular cab mRNA fragments to be translated among a pool of homologous mRNA (Flachmann and Kuhlbrandt, 1995).

2.II.d.5: Post-translational regulation of cab genes

In addition to regulation of cab at transcriptional and post-transcriptional levels, Cab protein accumulation is also regulated at the post-translational level. It has been noted that when plants are transferred to dark, chlorophyllide production stops but Cab protein synthesis continues for about 16 hours. However, the absolute levels of Cab proteins decline as a result of breakdown within the photosynthetic membranes (Bennett, 1981). Cab apoproteins are synthesized and transported to the chloroplast, and incorporated into thylakoid membranes. Unless there is sufficient chlorophyll a and b to stabilize Cab proteins, the Cab proteins break down and fail to accumulate despite high levels of cab mRNA (Cuming and Bennett, 1981).

These results suggest that chlorophyll a and b are required to stabilize Cab proteins and form LHC incorporated into thylakoid membranes. Since chlorophyll can only be produced in light, it is light, through the synthesis of chlorophyll a and chlorophyll b, that prevents turnover of light harvesting apoproteins in photosynthetic membranes (Bennett, 1981) and exerts a level of post-translational control over Cab protein accumulation.

2.II.d.6: Chlorophyll and Cab protein co-dependence

Just as Cab protein accumulation is dependent upon the presence of chlorophyll, chlorophyll accumulation appears to be dependent upon the presence of Cab proteins. Experiments conducted in 1989 reveal that when reaction core protein production is inhibited, there is a subsequent rapid reduction in accumulation of total chlorophyll (Maloney et al., 1989). Chlorophyll a and b deficiencies have been found to result from impaired integration of LHC apoproteins into thylakoids. LHCs are directly involved in synthesis of chlorophylls and some carotenoids. The final steps in chlorophyll a and b synthesis occur on LHC proteins in the thylakoid (Plumley and Schmidt, 1995). Rates of pigment synthesis appear to mirror rates of pigment accumulation and there is little futile pigment produced. Since Cab proteins degrade in the absence of chlorophyll, and chlorophyll cannot be produced in the absence of Cab proteins, Cab protein accumulation and chlorophyll accumulation are dependent upon each other (Plumley and Schmidt, 1995).

2.II.e: Development of Photosystem I

Within the chloroplast, Cab and other proteins are found within chlorophyll/protein complexes which can be divided into three classes; those associated with the reaction core of Photosystem I (PSI), those in the reaction core of Photosystem II (PSII) and those that serve light harvesting functions. Though each of these three systems contain chlorophyll/protein complexes, each has unique components working within them.

PSI is responsible for absorption of light energy, with maximum absorption capacity for wavelengths around 700 nm. PSI is embedded deep within the galactolipid bilayer of the thylakoid membrane and has a decidedly hydrophobic nature (Golbeck, 1992). PSI units have been found in great quantities in stromal, non-appressed regions of the thylakoid (Bennett, 1981).

Photosystem I can be divided into two components, the reaction core and the light harvesting antenna (LHCI). The reaction core of PSI is composed of a heterodimer of plastid encoded proteins from the psaA and psaB genes, which have molecular masses of 82-83 Kd and bind the chlorophyll a dimer (P700) and the initial chlorophyll a electron acceptor (Ao) (Green and

Durnford, 1996). The reaction core heterodimer contains approximately 100-140 chlorophyll a molecules (Golbeck, 1992). These additional chlorophyll a molecules act as a built in antenna for the reaction core (Green and Durnford, 1996).

There are four different nuclear encoded light harvesting pigment proteins associated with LHCI in higher plants. The proteins function to orient and precisely space their associated chlorophyll molecules in order to ensure efficient light energy transfer from the antenna to the reaction center (Dreyfuss and Thornber, 1994). The major protein components of LHCI are LHCIa (24Kd) and LHCIb (21-22 Kd). Each occur as trimers composed of very similar subunits (Thornber et al., 1993). According to Thornber et al. (1993), there are 3 LHCIb trimers and one LHCIa trimer for a total of 4 LHCIa/b trimers per LHCI complex. It has been determined that for each PSI reaction core, there are 8 LHCI molecules that are attached radially to the core complex (Boekema et al., 1990). It is expected that each LHCI binds 12-15 chlorophyll a molecules for a total of approximately 100 chlorophyll a molecules bound in the LHCI system per PSI reaction center (Golbeck, 1992). In experiments conducted on barley, LHCI molecules bound 21% of the total chlorophyll in the thylakoid membrane (Thornber et al., 1993).

2.II.f: Development of Photosystem II

Photosystem II is responsible for absorption of light energy, with maximum absorption occurring for wavelengths of 680 nm. As with PSI, PSII is also embedded within the thylakoid membrane (Burgi et al., 1987). However, unlike the stromal thylakoid location of PSI, PSII is found in greatest abundance in the appressed granal regions of the thylakoid membrane (Bennett, 1983).

Similar to PSI, PSII can also be divided into two components; the PSII reaction core and the associated light harvesting complex, LHCII. The PSII core complex consists of three chlorophyll/protein complexes; the reaction center, CP47 and CP43 (Green and Durnford, 1996). The reaction center consists of a pair of hydrophobic polypeptides, DI and D2, which are encoded by the plastid genes psbA and psb D. These proteins form a dimer that bind 4-6 chlorophyll a molecules, 2 pheophytins and 2 quinones. (Satoh, 1993, Seibert, 1993). CP47 and CP43 are 52 Kd and 48 Kd proteins encoded by the plastid genes, psbB and pcbC, respectively. Both proteins are very hydrophobic. It has been deduced that 20 chlorophyll a molecules and 4-5 β-carotene molecules are bound to each CP47 and CP43 polypeptide (Alfonso et al., 1994). CP47 and CP43 act as internal light harvesting antennae within the PSII reaction core.

The light harvesting complexes associated with PSII are referred to as LHCII. Although LHCII is predominantly associated with PSII, under certain light conditions, LHCII can act as a light antenna for PSI.

There are five different LHCII pigment proteins, the most abundant of which is LHCIIb. LHCIIb constitutes 1/3 of the total protein in the thylakoid membrane and binds 42% of total chlorophyll in the chloroplast (Peter and Thornber, 1991). Each LHCIIb subunit is associated with 2-3 xanthophyll molecules and 12-15 chlorophyll molecules, 8 of which are chlorophyll a molecules and 7 are chlorophyll b molecules (Butler and Kuhlbrandt, 1988, Kuhlbrandt and Wang, 1991). The remaining 4 LHCII pigment proteins have been labelled LHCIIa, c, d, and e. LHCIIa, c, and d each bind 3% of the total chlorophyll in the thylakoid while LHCIIe only binds 2% (Thornber et al., 1993). These last 4 LHCII complexes are considered minor LHCII subunits.

The organization of the different LHCII subunits around the PSII reaction core seems to follow a model in which the 4 minor LHCII particles are arranged in close proximity to the PSII reaction core and the abundant LHCIIb particles are comparatively further away from the reaction core (Dreyfuss and Thornber, 1994).

The LHCIIb subunit is a trimer , and 12 LHCIIb subunits are associated per PSII reaction center (Peter and Thornber, 1991). The LHCIIb trimer can vary in terms of its constituent polypeptides depending upon its proximity to the reaction center.

The structure of the 4 minor polypeptide complexes are thus far undetermined. It is not known whether they are organized as monomers, dimers, trimers, etc. However, it is known that there are one or two copies of each minor polypeptide per P680 reaction core.

A multimeric subcomplex of LHCIIa, c, e, d and a trimeric LHCIIb unit is found very close to the PSII reaction core (Thornber et al., 1993). This subcomplex is believed to connect energy flow from the bulk LHCIIb subcomplex to the reaction core (Peter and Thornber, 1991). The LHCIIb unit in this multimeric subcomplex contains 2 copies of the 28 Kd polypeptide and one copy of the 25 Kd polypeptide. As one moves to more peripheral antenna of PSII, the LHCIIb trimers consist of 28 and 27 Kd apoproteins rather than the 28 Kd and 25 Kd polypeptides of more internal LHCIIb units (Sigrist and Stahelin, 1994). This heterogeneity in LHCIIb complexes appears to reflect some differences in function. Peripheral LHCIIb units can undergo reversible phosphorylation in order to temporarily transfer light energy to either PSI or PSII depending upon light conditions. Internal LHCIIb units do not commonly undergo reversible phosphorylation (Bassi et al., 1988, Peter and Thornber, 1991). It is believed that the internal LHCII multimeric subcomplex is first assembled with the core complex to form a rudimentary PSII complex before the peripheral LHCIIb complexes can be added to the photosystem (Dreyfuss and Thornber, 1994).

2.II.g: Photosynthesis

Photosynthesis is the process used by plants to capture light energy and store that energy in a form that can be used by the plant to fix carbon and perform its life processes. The organelles that house the thylakoid membrane and the photosynthetic systems, PSI and PSII, are referred to as chloroplasts.

It is within the thylakoid membrane that photosynthesis occurs. Within the thylakoid membrane are four types of complexes that act to mediate light dependent vectorial electron flow and the resultant generation of ATP and reducing power. These four complexes include Photosystem I (PSI), Photosystem II (PSII), the cytochrome complex and ATP synthase. PSI and ATP synthase are predominantly found in the stromal thylakoid regions while PSII is found in greatest concentrations within granal thylakoid regions. Cytochrome complexes are found throughout the thylakoid system (Prezelin, B., Nelson, N., 1990).

The process of photosynthesis involves three steps that occur in the presence of light. These steps include absorption of light energy by the antenna, transfer of energy through the antenna to the reaction cores of the photosystems and the resulting electron transfer through PSI and PSII.

Only light in the violet to red (400-700 nm) region of the spectrum can be used for photosynthesis. Light of short wavelengths has higher excitation energy for photosynthesis than light of long wavelengths. Though the reaction centers of the photosystems are quite limited in the light wavelength they can directly use for photosynthesis, the addition of chlorophyll b, carotenoids and other pigments to the various LHCs allow the antenna to absorb a greater variety of light wavelengths.

When light energy is absorbed by an antenna chlorophyll molecule, the chlorophyll molecule is elevated to an excited state. The light energy is passed unidirectionally from one chlorophyll molecule to a neighboring chlorophyll molecule. As the light energy is passed between chlorophyll molecules, the excited state of the donor chlorophyll drops and the excitation state of the recipient chlorophyll is elevated. In this way, the light energy is passed unidirectionally from the antennae to the photosystem reaction cores (Prezelin, B., Nelson, N., 1990).

In PSII, the last phototrap for transfer of light energy is the chlorophyll a dimer, P680. At this point the absorbed light energy is used to split apart a water molecule to release an electron and O_2. This electron is transferred through a series of electron acceptors with varying redox potentials. The passage of the electron through the PSII electron transport chain causes the formation of a proton gradient across the thylakoid membrane. This proton gradient drives ATP formation via ATP synthase (Prezelin, B., Nelson, N., 1990).

Electrons flow from the electron donor side of PSII, through the cytochrome b_6f complex, to the electron acceptor side of PSI. In PSI, the phototrap chlorophyll a dimer in the reaction core is referred to as P700. Whereas PSII uses water as the electron donor, PSI uses plastocyanin as its electron donor. The light energized electron is passed to iron-sulfur protein electron acceptors (ferredoxin A and B) which, with the help of an oxidoreductase, reduce $NADP^+$ to NADPH. Both NADPH and ATP can be used in the dark reactions of photosynthesis to fix atmospheric CO_2 into carbohydrates usable by the plant (Prezelin, B., Nelson, N., 1990).

2.II.h: Regulation of photosynthesis

Photosynthesis occurs in green plants when they are illuminated. However, the quality of illumination can vary greatly. Trees with leaves in the top of a forest canopy receive light of different fluency and light quality than plants on the forest floor. These differences in illumination can affect the rates at which each of the photosystems function.

The reaction core of PSI (P700) preferentially absorbs wavelengths greater than 680 nm. The reaction core of PSII (P680) preferentially absorbs light of shorter wavelengths (Dennis and Turpin, 1990). If the light quality is skewed in one direction or the other, either predominantly above 680 nm or below 680 nm , one of the photosystems will be more active than the other. In order to keep light excitation energy balanced between the two photosystems and thus keep both photosystems working efficiently, a number of mechanisms have been developed.

For long term exposures to light conditions that favor one photosystem or another, plants can adapt by varying the PSI/PSII ratio, altering the arrangement of the thylakoid or changing the amount of light harvesting pigments serving each photosystem (Chow and Anderson 1987 a,b). In peas (*Pisum sativum*), transfer of plants from low to moderate irradiance was shown to result in doubling the photosynthetic capacity of leaves after seven days exposure. The cytochrome b_6f content of the leaves also doubled and there was a 35% increase in PSII (Chow and Anderson, 1987 a,b). Acclimation to higher irradiance allows optimal photosystem function and therefore allows the plant to avoid photoinhibition more effectively (Chow et al., 1991). Short term exposure to extreme light conditions can be withstood in plants by redirecting some absorbed light out of PSII into PSI or by rearranging the light harvesting complexes between PSI and PSII (Dennis and Turpin, 1990). Many of these adaptive changes, particularly the short term changes, are dependent on the redox state of cytochrome b_6f, the complex that links PSII to PSI in linear electron flow from water to $NADP^+$ (Anderson, 1992, Hauska et al., 1983).

Light that is predominantly below 680 nm will induce over-excitation of PSII relative to PSI. As electrons flow through PSII, the cytochrome b_6f complex accepts them and passes the electrons to PSI via plastocyanin. If PSII becomes overexcited as a result of the light regime, the cytochrome b_6f complex becomes very reduced. Located quite close to the cytochrome b_6f complex in the thylakoid is the LHCII kinase enzyme whose activity is governed by the redox state of cytochrome b_6f. Reduced cytochrome b_6f will activate the LHCII kinase. Activated LHCII kinase phosphorylates peripheral LHCII on the outer edges of grana (Anderson, 1992). The LHCII is phosphorylated on the surface-exposed N-terminal segment (Bennett, 1980) which enhances surface negative charge on the LHC. The repulsion experienced between individual phosphorylated LHCII is believed to drive them out of the stacked granal region (Bennett, 1983) into the stroma and closer to PSI. The LHCII, now in the stromal regions, act as antenna for PSI, funneling additional light energy to the PSI reaction core and thereby balancing the distribution of light energy between the photosystems. LHCII mediates appression of grana and once phosphorylated, LHCII move away from the grana causing reductions in membrane stacking by 10-15% (Biggins, 1982). This situation in which LHCII is phosphorylated and moved from grana to stroma is referred to as State II.

Light that is predominantly above 680 nm will induce over-excitation of PSI. This over-excitation will induce changes that will help redistribute the light energy away from PSI and toward PSII by inhibiting the transfer of light energy from PSII to PSI. Fewer electrons move through PSII resulting in oxidation of the electron carrier. The resulting redox state of cytochrome b_6f inactivates LHCII kinase (Anderson, 1992). A phosphatase system is believed to exist in stromal thylakoid regions. Once the stromal LHCII are dephosphorylated, the resulting lack of net negative charge allows migration of the LHCII back to the grana (Bennett, 1983). LHCII once again functions as antenna for PSII and increases the amount of light energy received by the PSII reaction core, thereby balancing the light energy distribution between PSI and II. The situation in which LHCII is located primarily in grana is referred to as State I. With the return of LHCII, granal stacking increases by 10-15%.

2.II.h.1: Summary

Plants are very capable of adjusting to their environment. In order to maintain the efficient functioning of both photosystems, plants are able to redistribute light energy and alter photosynthetic complexes as it becomes necessary. Plants have both long term modifications, such as varying PSI/PSII ratios, as well as short term modifications, including the temporary redistribution of LHCII between PSI and PSII. Such modifications allow the plant to function efficiently in a wide variety of light regimes.

2.II.i: Seed photosynthesis

Photosynthesis occurs in all green tissues and organs in higher plants. In addition to green leaves, stems and siliques, there is a fourth organ which is capable of photosynthesis. In *Brassica*, young seed accumulate chlorophyll and become photosynthetic as they develop. However, by the time the seed is mature it has lost its green pigment and is no longer able to photosynthesize. Seed act as carbon sinks during development, using photosynthates to produce the storage proteins, lipids and other compounds needed to produce mature seed. Despite the fact that seed photosynthesize, they are not the primary or most effective contributors of photosynthates aiding seed development.

Experiments conducted on *Brassica campestris* indicate that at the onset of flowering, the leaves are the primary sites of carbon dioxide assimilation and photosynthates are translocated to flowers, siliques and roots (Freyman et al., 1973). As development proceeds and senescence approaches, the leaves contribute less to seed development while the stems and siliques become an important source of photosynthates for young seed (Tayo and Morgan, 1975). During seed ripening, the siliques remain the principle sites of carbon assimilation and main source of photosynthates for maturing seed. As maturity progresses, the seed become an even stronger sink for photosynthates supplied by siliques. The siliques are likely the greatest contributor of photosynthates to seed development (Rood et al., 1994). Although these studies determined the photosynthetic contribution of various plant organs to seed development, they did not examine the photosynthetic contribution of the seed itself.

Experiments conducted in the late 1960's investigated the photosynthetic capabilities of developing pea seed and their importance to seed development (Flinn and Pate, 1970). Radiolabelled $^{14}CO_2$ was injected into the internal cavity of a pea pod and the sugars in the peas were subsequently measured for the incorporation of radioactive carbon. It was noted that the radiolabelled carbon dioxide was minimally assimilated into sugars by the contained seed. In order to eliminate the effect of a limited light supply within the pod, seeds were removed and subjected to full light. Analysis of the seed revealed that they failed to reassimilate more than 80% of the carbon dioxide lost through respiration (Flinn, 1969). These studies indicate that even in full sunlight, the developing pea seed is fixing insufficient quantities of CO_2 for development. Although this study was for *Pisum arvense* and may not apply wholly to the photosynthetic abilities of immature *Brassica napus* seed, the systems could be very similar.

Examination of the light that reaches the developing seed through the silique reveals that only 10% of the light reaching the silique is able to filter through to the seed (Green, per. comm). This small percentage of filtered light may prove to be a limiting factor in the photosynthetic actions of developing *Brassica napus* seed.

Light appears to serve another function in the developing seed, beyond its undetermined photosynthetic role. It has been suggested that light serves a stabilizing role for storage proteins in developing embryos. Experiments conducted by Nykiforuk examined the role of light in stabilization of storage proteins in haploid embryos of *Brassica napus*. In the absence of light, polypeptides of the seed storage protein, napin, failed to accumulate in haploid embryos. However, in the presence of light, napin did accumulate in examined embryos. The results of these studies indicate that light may act post-translationally to stabilize storage proteins in developing seed (Nykiforuk, 1996). Whether or not the same results would be obtained in the zygotic embryo remains to be seen, however, it is known that haploid embryos mimic many of the responses of zygotic embryos (Holbrook, L. et al., 1991).

2.III: DEGREENING

2.III.a: Methods of degreening

One of the final stages of seed development involves the controlled degradation and removal of green pigments and pigment/protein complexes from maturing seed. This process of degreening results in mature yellow canola seed. Degreening in plants is mediated by a number of different processes, each of which degrade chlorophyll in a different way to produce a variety of degradation products. The processes of degreening can be species dependent (Amir-Shapira et al., 1987), varying in the degradative processes as well as the stage during which degreening occurs.

Degreening occurs over a particular phase of seed maturation, depending upon the species involved. In canola (*Brassica napus*, cv. Westar) degreening appears to occur between 80% seed moisture and 45% seed moisture (Johnson-Flanagan and Spencer, 1996). Over this moisture range, chlorophyll and other pigment levels fluctuate (Johnson-Flanagan and Thiagarajah, 1990), enzyme activity in the thylakoid changes (Johnson-Flanagan and McLachlan, 1990 a,b), chlorophyll degradation product levels are altered (Johnson-Flanagan and Thiagarajah, 1990, Kennedy and Johnson-Flanagan, 1993) and the concentration of chlorophyll/protein complexes decreases (Kennedy and Johnson-Flanagan, 1993). There is still not a complete understanding of the processes involved in degreening, but several processes have been elucidated and other potential processes are still being investigated.

2.III.a.1: Chlorophyllase

One of the enzymatic systems for chlorophyll degradation involves the thylakoid membrane enzyme, chlorophyllase. Chlorophyllase is a protein dimer with component molecular weights of 29 and 33 Kd (Johnson-Flanagan and McLachlan, 1990a). Chlorophyllase acts at the first step in chlorophyll degradation by removing the phytol group from chlorophyll and pheophytin to form chlorophyllide and pheophorbide, respectively (Amir-Shapira et al., 1987).

In experiments with spinach leaves it was found that the chlorophyllide formed by chlorophyllase was derived predominantly from PSI antenna chlorophyll which is rich in chlorophyll a (Schoch and Brown, 1987). In both spinach (Schoch and Brown, 1987) and canola seed (Johnson-Flanagan and McLachlan, 1990a), chlorophyllase seems to prefer to hydrolyze chlorophyll a before chlorophyll b (Schoch and Brown, 1987). This suggests that chlorophyllase may have binding sites that preferentially bind chlorophyll a (Schoch and Brown, 1987).

Over the course of degreening, chlorophyllase activity and pigment levels change as seed moisture content changes. At 78% seed moisture, pigment content in the seed has reached maximum accumulation, with chlorophyll a levels being higher than chlorophyll b. At 80% seed moisture, chlorophyllase activity is low in the seed (Johnson-Flanagan and Spencer, 1996). As seed moisture content falls from 80% to 60%, chlorophyllide a levels increase significantly and peak at 70% seed moisture (Johnson-Flanagan and Thiagarajah, 1990). The chlorophyllide a levels remain quite high such that at 50% seed moisture, chlorophyllide a makes up ¼ of total pigment in canola seed. Peak chlorophyllase activity occurs at 75% seed moisture (Johnson-Flanagan and Spencer, 1996).

2.III.a.2: Peroxidase

Peroxidase is a second thylakoid bound enzyme found to function in degreening canola seed. The peroxidase enzyme is 54 Kd in size as determined by SDS-PAGE (Johnson-Flanagan and McLachlan, 1990b). The thylakoid peroxidase system is an H_2O_2 dependent, thylakoid bound, chlorophyll bleaching system (Matile, 1980). H_2O_2 , necessary for peroxidase activity, is produced by PSI (Mehler, 1951). While peroxidase is a fairly ubiquitous enzyme, the thylakoid bound peroxidase is associated specifically with chlorophyll degradation. Thylakoid-associated peroxidase is synthesized *de novo* during degreening. It has been noted that peroxidase-mediated degreening in maturing seed appears to be correlated with changes in ethylene production. Peroxidase activity appears to increase as silique ethylene levels rise during seed degreening (Johnson-Flanagan and Spencer, 1996).

Peroxidase-mediated degreening occurs at lower seed moisture content than does chlorophyllase-mediated degreening. Measured *in vitro*, peroxidase activity is fairly low at high seed moisture content, then increases as seed moisture reaches 68%, and attains maximum activity at 50% seed moisture (Johnson-Flanagan and Spencer, 1996). Though peroxidase activity is undeniably evident during seed degreening, peroxidase activity is quite low compared to chlorophyllase activity (Johnson-Flanagan and Spencer, 1996).

2.III.a.3: Mg-dechelatase

Another enzyme active during chlorophyll degradation is believed to catalyze one of the first steps in chlorophyll degradation in degreening canola seed (Johnson-Flanagan and Thiagarajah, 1990). The magnesium dechelatase system acts to remove the Mg^{2+} ion from the central ring structure of chlorophyll to produce the magnesium free molecule, pheophytin (Schoch and Vielwerth, 1983). The mechanism of action is not yet fully understood for the Mg-dechelatase system. Pheophytin levels are quite low in degreening seed, but do reach peak accumulation at 70% seed moisture (Johnson-Flanagan and Thiagarajah, 1990). The presence of both pheophytin and chlorophyllide in degreening canola seed indicates that there is more than one enzyme involved in chlorophyll degradation in degreening canola seed.

2.III.a.4: Chlorophyll oxidase

A fourth method of chlorophyll degradation makes use of an oxidative system of chlorophyll bleaching. Chlorophyll oxidase activity has been found in senescing leaf tissue (Schoch et al., 1984). Oxidase activity requires the presence of O_2 and activation by linolenic acid (Luthy et al., 1984), the latter being found in abundance in chloroplast membrane lipids. The primary breakdown product produced by chlorophyll oxidation is 13^2-hydroxychlorophyll a (Schoch et al., 1984) .

Chlorophyll oxidase activity varies for each of the photosynthetic components in the thylakoid. Photosystem II has the highest chlorophyll oxidase activity, followed by fairly high activity in isolated thylakoids, low activity in PSI and no chlorophyll oxidase activity in LHC. Bleaching of chlorophyll by chlorophyll oxidase appears to be a property of the photosystems themselves, but the catalytic process is unlikely to be mediated by a specific oxidation enzyme (Luthy et al., 1986).

Though chlorophyll oxidase activity has been found in senescing leaf tissue of manipulated barley thylakoid components, the role of chlorophyll oxidase in chlorophyll catabolism *in vivo* has not been established (Luthy et al., 1986). A role for chlorophyll oxidase in the degreening of canola seed has also not been determined.

2.III.a.5: Free radical chlorophyll degradation

There is also a non-enzymatic chlorophyll degradation system that appears to involve free radicals (Sakaki et al., 1983). Free radical degradation of chlorophyll is believed to require both light and oxygen (Ziegler and Schanderl, 1969). Although no intermediates of non-enzymatic chlorophyll degradation have been found, it has been suggested that the macrocyclic ring in chlorophyll might be linearized by photooxidation to form intermediate tetrapyrroles and monopyrroles. Further photooxidation might lead to simple molecules, such as NH_3 or CO_2 (Hendry et al., 1987).

2.III.a.6: Summary

It is possible that only a few of the described chlorophyll degradation pathways are active during seed degreening. Evidence has been found for the activity of chlorophyllase, Mg-dechetalase and peroxidase during seed degreening due to the presence of chlorophyllide, pheophytin, chlorophyllase activity and peroxide activity (Johnson-Flanagan and Spencer, 1996). The process of seed degreening, through the myriad of chlorophyll degradation systems available, results in the transformation of a green canola seed into a yellow seed.

2.III.b: Effect of sub-lethal frost on degreening - production of green seed

Very often, particularly in cooler growing climates, such as regions of northern Alberta, the immature green canola seed will fail to lose its green pigments, resulting in mature canola seed which remains green. One of the causes of green seed is sub-lethal freezing, which perturbs the normal maturation process in canola seed by inhibiting seed degreening, inducing renewed pigment synthesis, altering the activity of enzymes such as chlorophyllase and peroxidase (Hodgins et al., 1989, Johnson-Flanagan et al., 1990, Johnson-Flanagan and McLachlan, 1990a,b) and hastening dessication (Johnson-Flanagan et al., 1991). The alteration of enzyme activity and other frost induced changes are generally dependent upon the minimum temperature reached and the seed moisture content at the time of freezing (Johnson-Flanagan et al., 1991).

During normal maturation the seed requires 35 days from the point of maximum seed moisture to reach the mature seed moisture content of 11%. However, after a sub-lethal frost, the seed undergoes very rapid desiccation and reaches mature seed moisture content in 7-10 days after frost (Johnson-Flanagan et al., 1991). With this rapid desiccation, there is also an accumulation of storage proteins (Johnson-Flanagan et al., 1991) and LEA (late embryogenic abundant) proteins in the seed, suggesting that not only is desiccation very rapid, but maturation of the seed is hastened (Johnson-Flanagan et al., 1992). The rapid shift to desiccation may deprive the seed of the time required to clear chlorophyll.

Sub-lethal freezing also inhibits photosynthesis. Freezing is known to disrupt PSI and PSII activity (Grafflage and Krause, 1986). In tomato leaves, 67% of the frost-induced photosynthetic inhibition is a result of impaired biochemical processes of the chloroplast while 33% of inhibition is the result of increased stomatal resistance to gas exchange (Cooper and Ort, 1987). Disruption of photosynthetic activity interferes with breakdown processes (Woolhouse, 1987).

A single frost has the potential to lead to a green seed problem. Although mature green seed generally contains a high proportion of chlorophyll and pheophytin with low amounts of dephytylated pigment (Johnson-Flanagan, 1992), seed moisture content at the time of freezing and minimum temperature attained can alter the pigment composition and seed development of the resulting seed. Generally, the pigment content in the mature seed increases as minimum temperature decreases (Johnson-Flanagan, 1992). Mature seed frozen between 78%-67% seed moisture (high seed moisture) have high levels of chlorophyll and pheophytin, and over 1/3 of the pigments are dephytylated. A small proportion of pigments are associated with the thylakoid. In mature seed that have been frozen at intermediate seed moisture content (67%-60%) there is less pigment associated with complexes and there is more free chlorophyll in the seed compared to seed frozen in the 70% moisture range (Johnson-Flanagan et al., 1990). Mature seed frozen at low seed moisture content (59-50%) contain chlorophyll and pheophytin. Less than 1/5 of the total pigments are dephytylated. The pigment that is present is highly complexed to Cab proteins (Green et al., 1995).

In addition to pigment retention, freezing at low to intermediate seed moisture contents leads to significant increases in pigment content in mature seed. There are particularly large increases in chlorophyll and pheophytin (Johnson-Flanagan et al., 1990). The pigment increases are associated with increases in Cab proteins (Green et al., 1995).

Freezing also influences the function of the pigment degrading enzymes, chlorophyllase and peroxidase. Chlorophyllase activity was measured 24 hours after sub-lethal freezing to -5° C and no change in chlorophyllase activity was detected (Johnson-Flanagan and McLachlan, 1990a). However, 4 days after freezing, both chlorophyllase accumulation and activity increased 280%. Thus, failure of seed to degreen following a sub-lethal frost does not appear to result from a reduction in chlorophyllase activity.

Freezing dramatically affects the chlorophyll bleaching ability of thylakoid-bound peroxidase. Following a sub-lethal frost, peroxidase activity in maturing seed decreases significantly (Johnson-Flanagan and McLachlan, 1990b). The enzyme is still present in the thylakoid but there is almost complete lack of activity. Inhibition of peroxidase activity following freezing may be responsible in part for lack of seed degreening (Johnson-Flanagan, 1992).

2.IV: APPROACHES TO ALLEVIATING THE GREEN SEED PROBLEM

There are a number of approaches to reduce chlorophyll content in green seed. These include hastening chlorophyll degradation, inhibiting chlorophyll synthesis or reducing chlorophyll accumulation in the seed.

2.IV.a: Hastening chlorophyll degradation

It may be possible to hasten chlorophyll degradation in the seed by increasing the activity of one of the enzymes involved in chlorophyll degradation. By upregulating a chlorophyll degradation enzyme, more enzyme will be available to aid in the degradation of seed chlorophyll.

Chlorophyllase is the main chlorophyll degradation enzyme involved in canola seed degreening (Johnson-Flanagan and Spencer, 1996) and is a promising candidate for upregulation. Normally peak chlorophyllase activity occurs at 65% seed moisture (Johnson-Flanagan and Spencer, 1996). By using a series of frost inducible seed specific promoters to drive the chlorophyllase gene, it may be possible to increase chlorophyllase activity over a broad range of seed moisture contents. It also may be possible to initiate chlorophyll degradation long before the seed normally begins to degreen.

Chlorophyllase has certain qualities which make it a promising subject for upregulation. As mentioned above, chlorophyllase is the most active enzyme involved in chlorophyll degradation in canola seeds. Increasing chlorophyllase activity should result in higher levels of chlorophyll degradation. Another quality of chlorophyllase is its ability to resist inhibition by sub-lethal freezing. Sub-lethal freezing actually increases chlorophyllase activity by 280% several days after the frost (Johnson-Flanagan, 1992). Increasing the expression of chlorophyllase genes should result in very high levels of chlorophyllase activity after a frost, since endogenous chlorophyllase activity will increase. One would expect that by establishing very high levels of chlorophyllase activity, the rates at which chlorophyll is degraded would increase. However, there are a number of factors that may limit the effectiveness with which upregulated chlorophyllase increases chlorophyll degradation. Chlorophyllase activity is fairly high throughout seed maturation, as is evident in the increasing levels of dephytylated pigments as seed moisture content decreases. Since chlorophyllase activity is already high, upregulating chlorophyllase expression may have no impact on rates of chlorophyll degradation as chlorophyllase activity may not be the limiting factor in chlorophyll degradation. Evidence for this may be found after a sub-lethal frost when chlorophyllase activity increases by 280% but chlorophyll levels fail to decrease.. Further to this, chlorophyllase activity was not found to be associated with degreening in *Brassica juncea*, a species known for its lack of green seed.

2.IV.b: Inhibiting chlorophyll synthesis

In order to reduce chlorophyll content in the seed, it may be possible to inhibit chlorophyll synthesis, thereby preventing chlorophyll from establishing itself in the seed. One method which might be used to reduce chlorophyll synthesis could be suppression of one of the enzymes in the chlorophyll synthesis pathway. By suppressing the activity of a chlorophyll synthesis enzyme, the production of chlorophyll would be reduced, if not completely suspended, preventing chlorophyll from accumulating in the seed. An advantage of down-regulating the production of chlorophyll synthesis enzymes is that many of the enzymes are nuclear encoded and the genes have been sequenced (von Wettstein et al., 1995). With this information, it should be possible to suppress the genes encoding chlorophyll synthesis enzymes using antisense technology. Antisense technology will be discussed later in this review.

There are many factors that limit the use of enzyme inhibition for reducing chlorophyll synthesis. Some of the enzymes used for chlorophyll synthesis are also used for production of other compounds required by the plant. For instance, the enzymes of the C5 pathway are required for the production of both chlorophyll and heme. Heme is involved in respiration in plants. The biosynthetic pathways of heme and chlorophyll do not diverge until protoporphyrin IX (Tanaka et al., 1996). In order to ensure that the plant will not lose it supply of heme, none of the enzymes involved in production of protoporphyrin IX can be suppressed.

Another factor which limits the use of enzyme inhibition for reducing chlorophyll synthesis is the formation of photosensitive intermediates. When an enzyme becomes inhibited, its substrate begins to accumulate. If the accumulating intermediate is photosensitive, it can react in the cell to cause photodynamic damage to the plastid. For example, if protochlorophyllide reductase is inhibited, protochlorophyllide begins to accumulate in the plastid. The accumulation of protochlorophlyllide causes damage to the plastid. If enough damage is incurred, the plant will die (von Wettstein et al., 1995). Therefore, the reactivity of intermediates is of primary concern when considering whether or not enzyme activity should be suppressed.

2.IV.b.1: Summary

Inhibiting chlorophyll synthesis enzymes to reduce chlorophyll accumulation is accompanied by many limitations. In order to maintain heme synthesis, inhibition of chlorophyll synthetic enzymes that function before the formation of protoporphyrin IX cannot be considered. Beyond protoporphyrin IX, the photosensitive reactivity of chlorophyll intermediates must be carefully scrutinized before suppressing any of the chlorophyll synthesizing enzymes. Considering the limitations involved with inhibiting a chlorophyll synthesizing enzyme, a third method of reducing chlorophyll accumulation in canola seed may prove to be more promising.

2.IV.c: Reducing chlorophyll accumulation

An effective means of reducing chlorophyll accumulation may prove to be the reduction of Cab protein levels within the seed. It is known that chlorophyll is able to accumulate by binding to Cab proteins in chlorophyll/protein complexes in the thylakoid. In the absence of chlorophyll, Cab proteins are destabilized and degrade (Bennett, 1983). Similarly, in the absence of Cab proteins, chlorophyll does not accumulate (Plumley and Schmidt, 1995). Therefore, chlorophyll and Cab proteins stabilize each other and are codependent. If the amount of Cab protein in the seed is reduced, a corresponding reduction in chlorophyll accumulation should occur.

The gene encoding LHCIIb, major protein of LHCII, has been sequenced from *Brassica napus* and can be used to develop an antisense gene. Expression of the antisense cab gene should repress the accumulation of the LHCIIb protein in transgenic canola plants. In order to ensure the antisense cab is expressed only in the seed, the antisense gene would require a seed specific promoter. A number of seed specific promoters such as napin and oleosin have been isolated (Kridl et al., 1991, Lee et al., 1991) and could be used to direct antisense cab expression. Compared to the methods of hastening chlorophyll degradation or inhibiting chlorophyll synthesis, using antisense technology to reduce the accumulation of Cab proteins may be the most effective means of limiting chlorophyll accumulation and reducing the occurrence of green seed.

2.V: TRANSFORMATION

Transformation of plant tissues has become very popular in recent years as a means of inserting foreign genes into plant genomes to produce specific phenotypic traits. Techniques, such as electroporation and particle bombardment, have become quite popular for transforming protoplast cells. However, the method of choice for transforming larger explants, such as leaf discs or petioles, has been *Agrobacterium tumefaciens*-mediated transformation.

2.V.a: *Agrobacterium tumefaciens* and the Ti plasmid

Agrobacterium tumefaciens is a soil bacterium that is responsible for the development of crown gall disease in dicotyledonous plants (Watson et al., 1975). *Agrobacterium tumefaciens* acts as a plant pathogen by infecting wounds on plant stems causing the formation of tumors. Upon infection, the bacterium transfers a fragment of its own DNA to the plant cell, where it is incorporated into the plant genome. The infected plant cells then produce special compounds encoded by the transferred DNA. These compounds are metabolized by the bacteria. *Agrobacterium* infection represents the only known naturally occurring example of genetic engineering in the plant kingdom (Cangelosi et al., 1991). Scientists have attempted to take advantage of the natural gene transfer ability of *Agrobacterium* in order to develop a vector system for inserting foreign genes into various plant genomes.

Agrobacterium tumefaciens contains two separate bodies of genetic material; the bacterial chromosome and the tumor-inducing (Ti) plasmid. While the bacterial chromosome determines the host range of infection, the Ti plasmid is responsible for tumor production (Garfinkel and Nester, 1980). The Ti plasmid, which can be at least 100 megadaltons, is divided into two regions; the transfer DNA (T-DNA) region and the virulence (vir) region (Garfinkel and Nester, 1980).

T-DNA is the part of the Ti plasmid that is transferred and integrated into the genome of the infected plant cell. The T-DNA gene products are responsible for formation of tumor cells and synthesis of the special amino acids, opines, that are catabolized by the *Agrobacterium* (Murai and Kemp, 1982, Petit and Tempe, 1978). The T-DNA, which is about 23 Kb in length (Lemmers et al., 1980) is flanked on the right and left sides by 25 base pair (bp) direct repeated sequences (Zambryski et al., 1982). The 25 bp direct repeats at the right border are essential for transfer of T-DNA from the Ti plasmid into the plant genome (Wang et al., 1984).

The vir region of the Ti plasmid carries over 20 virulence genes. The products of the vir genes are responsible for catalyzing the transfer of T-DNA from the Ti plasmid to the plant cell (Cangelosi et al., 1991). It is only through the combined expression of virulence genes and T-DNA genes that crown gall tumors can form.

2.V.b: Modification of the Ti plasmid

In order for scientists to take advantage of the DNA transfer ability of *Agrobacterium*, the Ti plasmid needed to be modified. The modified Ti plasmid had to possess two characteristics; the plasmid had to allow DNA transfer to plant cells without affecting normal plant growth, and it had to allow foreign DNA to easily be inserted between terminal sequences flanking the T-DNA (Caplan et al., 1983). In order to fulfill the listed requirements, a number of alterations were made to the Ti plasmid.

One modified Ti plasmid that fulfilled the listed requirements was pGV3850, a nononcogenic derivative of the nopaline Ti plasmid C58. The T-DNA genes responsible for tumor formation were removed. The cloning vehicle, pBR322, was also inserted into the T-DNA to serve as a region of homology for recombination to introduce foreign DNA (Caplan et al.,1983).

The first successful attempt to introduce foreign genetic material into plant cells made use of the modified Ti plasmids, pTiT37 and pTiAGNC. The prokaryotic transposons, Tn5 and Tn7, were inserted into the T-DNA of pTiAGNC and pTiT37, respectively (Garfinkel et al., 1981, Hernalsteens et al., 1980). The T-DNA carrying the transposons were effectively transferred into the plant genome. Unfortunately, the genes encoding the transposons were not expressed, indicating that additional improvements to the Ti plasmid system were required.

It was believed that the transposons in the above experiment were not expressed in the plant because the eukaryotic cells did not recognize the prokaryotic promoter sequences. A number of promoter sequences have subsequently been added to T-DNA and found to be effective in inducing transcription of foreign genes, including mammalian, bacterial, and fungal genes, in transformed plant cells (Caplan et al., 1983). The nopaline synthase gene promoter (nos 5') contains a TATA box and AGGA sequence necessary for transcription in plant cells. In the first successful example of expressing foreign DNA in a plant cell, nos 5' was effective in promoting transcription of the bacterial chloramphenicol acetyltransferase gene (Herrera-Estrella et al., 1983). The promoter of the cauliflower mosaic virus (CaMV 35S) (Moloney et al., 1989) and the promoter of the mannopine synthase gene (mas 5') (McBride and Summerfelt, 1990) have also been used effectively for driving heterologous gene expression in transformed plant cells. The termination sequences, nos 3' and mas 3' are commonly used to terminate transcription of foreign genes in transformed plants (Herrera-Estrella et al., 1983).

In addition to the development of effective promoter sequences, many other alterations have been made to the Ti plasmid. The addition of insertional inactivation markers, selectable and detectable markers, high copy number origins of replication and the development of binary vector systems were intended to improve the technique of plant transformation.

2.V.c: Addition of multiple cloning sites

One improvement made to Ti plasmids was the addition of a multiple cloning site combined with an insertional inactivation marker in the T-DNA. The lac Z' gene, derived from *Escherichia coli* (Yanesch-Perron et al., 1985) contains a series of unique restriction sites and acts as a multiple cloning site when inserted in the T-DNA. The protein from the lac Z' gene produces a blue color when the bacteria are grown in the presence of X-gal. When foreign DNA is inserted into the lac Z' multiple cloning site, the lac z' gene is disrupted and the blue color is no longer produced in the bacteria. It becomes possible to differentiate between bacterial colonies containing foreign DNA (white) and bacterial colonies without foreign DNA (blue) (Bevan, 1984).

2.V.d: Selectable and detectable markers

Selectable and detectable markers have also been added to the Ti plasmid in order to help identify the presence of plasmid or foreign DNA in both bacterial and plant cells. It is important to actively maintain the Ti plasmid in bacteria. Bacteria grown in non-selective conditions will often spontaneously lose the Ti plasmid. Antibiotic resistance markers located outside the T-DNA region, such as gentamycin resistance (Hirsch and Beringer, 1984) are now a common feature of Ti plasmids. When bacteria are grown in the presence of the antibiotic, only those bacteria containing Ti plasmids with the resistance gene will grow. In this way, bacteria containing the Ti plasmid are maintained.

It is also important to have a means of differentiating between transformed plant cells and untransformed plant cells. It has become a standard practice to link an antibiotic resistance gene to the foreign genes that are to be transferred to the plant genome. One of the most common antibiotic resistance genes incorporated into the T-DNA as a selectable marker is the neomycin phosphotransferase II gene (nptII) (Houch et al., 1988). The nptII gene confers kanamycin resistance to transformed plant cells. Non-transformed shoots will bleach and die in the presence of kanamycin while transformed shoots will remain green and continue to grow. In this way, nptII allows for selection of transformed shoots on kanamycin-enriched media.

A method has quite recently been developed for identification of transformed plant cells that does not involve antibiotic resistance. Instead of inserting an antibiotic resistance gene into T-DNA, the *Escherichia coli* uid A gene is inserted. The uid A gene encodes the β-glucuronidase (Gus) enzyme. Activity of the Gus enzyme can be detected spectrophotometrically, fluorometrically and histochemically. In histochemical detection, the Gus enzyme acts upon the substrate 5-bromo-4-chloro-3 indolyl glucuronide (X-gluc) to produce a blue precipitate. In transformed plant cells, Gus activity is detected by the formation of blue pigment in the tested tissues, while non-transformed cells do not produce this blue pigment (Jefferson, 1985). Since

Gus activity does not affect the growth of the regenerating shoot, the uid A gene is considered a detectable , rather than a selectable, marker.

2.V.e: Organization of T-DNA components

In order to use insertional inactivation, and selectable and detectable markers effectively, the components of engineered T-DNA must be arranged in a particular order. T-DNA transfer begins at the right T-DNA border and moves toward the left . The lac Z' gene containing the multiple cloning site should be located very close to the right border. To the left of the lac Z' gene, selectable and detectable markers can be inserted. With this organization of T-DNA components, the foreign genes inserted into the multiple cloning site will be transferred to the plant genome before the selectable and detectable markers. Expression of the selectable markers in transformed plant cells can then be considered indicative of the presence of the foreign genes in the plant genome.

2.V.f: Replacing the origin of replication in the Ti plasmid

A fourth set of improvements made to Ti plasmids involved replacing the original Ti plasmid origin of replication (ori) with an origin of replication that confers high stability and high copy number in *Escherichia coli.* When designing the T-DNA to be inserted into the plant genome, most of the genetic manipulation of the T-DNA occurs in *Escherichia coli.* Unfortunately, in the past, the Ti plasmid did not naturally replicate in *Escherichia coli* making it necessary to subclone part of the T-DNA into an *Escherichia coli* cloning vehicle before the T-DNA could be engineered with foreign genes (Barton and Chilton, 1983). In order to allow manipulation of the Ti plasmid directly in *Escherichia coli,* the naturally occurring ori has been replaced with origins of replication that confer increased copy number in *Escherichia coli.* The ColEI origin of replication, derived from the pBr322 plasmid, has been inserted into the Ti plasmid, pCGN 1532 (McBride and Summerfelt, 1990). The ColEI ori allows replication of a high copy number of Ti plasmids to accumulate in *Escherichia coli.* The establishment of high copy numbers of Ti plasmid allows a high recovery of plasmid DNA from mini-preps and consequently easier manipulation of T-DNA.

2.V.g: Binary vector systems

Perhaps the most important modification of the Ti plasmid has been the development of the binary vector system. Early transformation experiments with *Agrobacterium* required that the T-DNA segment engineered in *Escherichia coli* be reintroduced into the Ti plasmid by virtue of homologous recombination (Barton and Chilton, 1983). It was believed that it was necessary to have both the T-DNA and the virulence region located on the same plasmid in order to induce T-

DNA transfer to the plant genome. However, it has been revealed that the products of the vir region can act *in trans* to induce transfer of T-DNA located on a separate Ti plasmid (Hoekema et al., 1983). The task of transferring T-DNA can be divided between two Ti plasmids, one housing the virulence genes and the other housing the T-DNA.

With all of the improvements made to Ti plasmids, it has become possible to engineer the T-DNA region of a small disarmed Ti plasmid in *Escherichia coli* and transfer the Ti plasmid to *Agrobacterium*. Once the disarmed Ti plasmid is in the *Agrobacterium*, the small resident virulence plasmid can induce transfer of the T-DNA from the disarmed Ti plasmid to the plant genome. The use of binary vectors has eliminated the need for homologous recombination into the Ti plasmid T-DNA region. Binary vectors, which have become more compact and enhanced with various oris, multiple cloning sites and selectable markers, are a simple and effective tool for introducing foreign DNA into plant genomes (McBride and Summerfelt, 1990).

2.VI: ANTISENSE TECHNOLOGY

Antisense RNA has become a popular tool for manipulating the expression of genes *in vivo.* An antisense gene is simply an inverted complementary copy of a gene or segment of a gene. The RNA from the antisense gene is complementary to the mRNA of the original gene. The antisense and sense RNAs are expected to bind one another to form an RNA duplex. This RNA duplex formation somehow blocks expression of the original gene. The study of both natural and artificial antisense mRNAs have allowed researchers to suggest a number of mechanisms through which antisense RNA inhibits gene expression.

2.VI.a: Prokaryotic antisense

Antisense RNAs were originally recognized in prokaryotes as a natural means of regulating gene expression (Simons and Kleckner, 1983). It has been suggested that naturally occurring antisense genes may have been formed by reverse transcription of a gene to form a cDNA which became integrated back into the genome (Inouye, 1988). Antisense RNAs were found to repress gene expression in prokaryotes at the levels of translation or transcription, depending upon the antisense RNA species and the gene being repressed (Inouye, 1988). For instance, pOUT RNA acts to bind transposase mRNA and inhibits translation of transposase RNA (Simons and Kleckner, 1983). However, tic RNA inhibits transcription of the crp gene, which encodes the cAMP receptor protein (Okamato and Freundlich, 1987).

2.VI.b: Proposed mechanisms of antisense action

Although the precise mechanisms of gene inhibition are not fully understood, researchers have suggested three possible classes of antisense action. Classes I and II involve inhibition of translation while Class III involves inhibition of post-transcriptional processes (Hiatt et al., 1989, Inouye, 1988, Lichtenstein, 1988).

The mechanism of Class I antisense RNA involves processes in the cytoplasm that inhibit translation. When sense and complementary antisense mRNAs are transported from the nucleus to the cytoplasm, the complementary RNAs bind within the cytoplasm to form an RNA duplex. If the RNA duplex covers the ribosome binding site of the mRNA, ribosomes cannot bind and translation does not occur (Coleman et al., 1985).

The mechanism of Class II antisense RNA action involves RNA duplex formation in the nucleus. Antisense/sense RNA duplexes which form within the nucleus cannot be transported out of the nucleus. In this way, sense mRNA cannot be transported or translated (Kim and Wold, 1985).

The final proposed mechanism for antisense RNA action involves inhibition of post-transcriptional processes. It is believed that antisense/sense RNA duplexes formed within the nucleus are very unstable and degrade rapidly (Crowley et al., 1985).

2.VI.c: Designing artificial antisense RNA

When designing an antisense RNA, a number of factors must be taken into consideration. The target gene to be suppressed must be specified, the desired length and sequence of the antisense RNA fragment must be determined, and the promoter used to drive the antisense construct must be chosen.

When choosing the gene to be targetted, it is important that the chosen gene imparts a visible phenotype on the plant. In this way, when gene expression is inhibited by antisense, the effects of the antisense gene can be scored. Molecular analysis in cases of gene inhibition by antisense may not reveal dramatic changes in nucleic acid levels (van der Krol et al., 1990). For instance, Stockhaus found that plants transformed with an antisense gene showed no reduction in target RNA and there was almost no detectable antisense RNA, but the plant did express an antisense phenotype (Stockhaus et al., 1990).

Antisense RNAs can be applied to any mRNA as long as the nucleotide sequence of the target gene is known (Inouye, 1988). However, when designing the antisense RNA construct, the desired length of the antisense RNA must be determined. It has been theorized that by designing an antisense fragment to cover a particular section of the sense mRNA, gene inhibition will occur through different mechanisms. By designing an antisense RNA that covers the 5' end of the sense mRNA, capping of the 5' end is inhibited. Similarly, designing an antisense fragment which covers the 3' poly A mRNA site should inhibit the addition of the poly A tail. By inhibiting the processing of sense mRNA the transport of the mRNA from the nucleus to the cytoplasm should be impeded (Inouye, 1988).

Despite the above theories, designing an effective length for antisense RNA is not straightforward. Experiments conducted by Flachmann and Kuhlbrandt showed that a 105 nucleotide antisense construct was as effective as a 550 nucleotide antisense fragment for inhibiting the expression of an LHCII protein gene (Flachmann and Kuhlbrandt, 1995). Experiments conducted by Sandler et al. in 1988 revealed that an 110 bp antisense nos fragment was more effective than a 373 nucleotide sequence or the full length antisense gene in repressing expression of a nopaline synthase gene (Sandler et al., 1988). The length of the most effective antisense fragment does not appear to be consistent among different genes and usually must be empirically determined for each gene. It has been suggested that the success of antisense suppression of genes is dependent upon expressing the appropriate region of the gene in antisense orientation. This requirement may reflect the effect of mRNA folding and stability on hybridization of sense and antisense RNAs.

2.VI.d: Promoters for antisense genes

Another point of consideration when designing an artificial antisense RNA is the choice of promoter used to drive the antisense. The choice of promoter is dependent upon when and where the antisense gene should be expressed. If the antisense is to be expressed throughout the plant, a constitutive promoter is desirable. It is believed that a strong promoter is necessary for achieving high expression of antisense RNA, and excess amounts of antisense RNA are required for effective reduction of target mRNA (Inouye, 1988, Hiatt et al., 1989). One of the first constitutive promoters used to drive antisense was the nos 5' promoter (Sanders et al., 1987) derived from the nopaline synthase gene from *Agrobacterium.* CaMV 35S also functions as a constitutive promoter (Moloney et al., 1989). The 35S promoter is thirty fold stronger in driving construct expression than is the nos promoter, making 35S a very popular promoter (Sanders et al., 1987).

2.VII: TISSUE SPECIFICITY

When inserting foreign genes into plant tissues, it is important to ensure that the gene being inserted is expressed in the appropriate plant organ and at the appropriate developmental stage. This targeting of genes to particular organs and developmental stages is referred to as tissue and temporal specificity. Directing the expression of inserted genes to appropriate tissues is most often accomplished through the use of various promoter sequences. If the foreign gene is to be expressed in a particular tissue, such as the root or the seed, a tissue specific promoter must be chosen.

There are a number of promoters that target gene expression to the seed. Napin and oleosin are two seed storage proteins whose promoters have been isolated and used to direct expression of foreign DNA to the seed (Kridl et al., 1991, Lee et al., 1991). One example of tissue specific gene expression involved experiments conducted by Knutson in 1992. The experiments focused on altering lipid metabolism in *Brassica rapa* and *Brassica napus*. The genetic alterations were to be limited to the oil storing embryo in order to prevent disrupting lipid biosynthesis required for membrane structures in other plant tissues. In order to target the antisense stearoyl ACP-desaturase gene to the seed, the antisense gene was driven by the promoter from the *Brassica rapa* napin gene, BcNaI. Napin gene expression is first detectable in the seed at 17 days after pollination (DAP) and reaches maximum expression between 21-30 days after pollination (Kridl et al., 1992). With the napin promoter driving the antisense gene, a reduction in desaturase activity was noted at 26 DAP, a stage of high enzyme activity (Knutson et al., 1992). So not only was the napin promoter effective in directing gene expression to the seed, it was also effective in directing gene expression to a specific developmental stage.

When manipulating a gene that is to be expressed only during a particular developmental stage, it is of limited benefit to have that gene expressed randomly during development. For instance, in the above example of antisensing the stearoyl ACP-desaturase gene, the desaturase enzyme is most active around 26 DAP (Knutson et al., 1992). It would be ineffective to express the antisense gene when the sense gene is not being transcribed. The periods of activity of the antisense and sense genes must overlap in order to get effective reduction in target gene expression, as in the overlap of high desaturase activity and napin promoter activity.

There are qualities innate to some promoters which limit their usefulness in directing tissue specific gene expression. Promoters isolated from monocotyledonous plants often do not function when transformed into dicotyledonous plants. Experiments conducted by Lee et al. (1991) revealed that the monocot maize oleosin gene driven by the maize oleosin promoter was not expressed in transformed dicot *Brassica napus*, but the same monocot gene driven by a *Brassica napus* napin promoter was abundantly transcribed. These results suggest that in order to ensure effective transcription of inserted genes, it is best to use dicot promoters in dicot plants and monocot promoters in monocot plants.

2.VIII: PROBLEMS WITH TRANSFORMATION

The process of inserting foreign genes into plants via *Agrobacterium* has been one of the most exciting discoveries in science in the last twenty five years. The evolution of binary vector systems and development of improved transformation methods have led to huge volumes of research on the topics of gene regulation and developmental processes. However, despite the success and popularity of plant transformation, the process is afflicted with a few negative side effects.

One of the first noticeable effects of plant transformation is the development of altered morphology in the primary transgenics. Unusual leaf morphology and altered floral structure are two of the most common visual alterations. Elongated pistils in the floral structure often result in poor seed set (Radke et al., 1988). Other observed morphological variations include pollen sterility and absence of stigma and style (personal observation). These altered morphologies, referred to as somaclonal variations, have also been observed in non-transformed plants derived from plant cells cultured *in vitro* (Radke et al., 1988) and are likely the result of tissue culture stress. The development of somaclonal variation in transgenics makes it difficult to assess the effect of inserted foreign genes on the morphology of the plant. Fortunately, somaclonal variation is prevalent only in the primary transgenics and its effects are rarely observed in subsequent generations (Radke et al., 1988).

Another problem experienced by transgenic plants is the phenomenon of gene silencing (Matzke and Matzke, 1995). It has been noticed over the last ten years that foreign genes inserted into plant genomes are often inactivated in subsequent generations. In gene silencing, the genes are still present in the genome, but expression has been inhibited in some way.

Three mechanisms have been proposed to explain transgene silencing based upon different nucleic acid interactions. Transgenic DNA-DNA interactions and transgenic DNA-endogenous RNA interactions are believed to lead to *de novo* methylation of foreign genes. Methylated genes often are not transcribed (Matzke and Matzke, 1995). The third mechanism involves the additive effects of RNA from an endogenous gene and RNA from a homologous transgene. It is believed that when RNA accumulates to a threshold level, an RNA degradation system is activated that degrades the elevated RNA species, essentially inactivating expression of the endogenous gene and the transgene. This RNA-RNA mechanism is also referred to as co-suppression (Jorgensen, 1992). Gene silencing has become a serious problem associated with plant transformation because the genes inserted are prevented from being expressed, essentially undermining the entire goal of plant transformation.

Concerns have also been raised regarding the environmental effects of transformation. One of the greatest concerns involved the spreading of undesirable genes into native populations by outcrossing. For instance, a transgenic plant line containing genes responsible for herbicide resistance may cross with native plant populations resulting in herbicide resistant native plant. Situations such as this may cause reductions in crop yield as a result of competition between the crop and weeds which are no longer controlled by herbicide application. There is currently much research to promote sexual sterility in transformed organisms if they are not to be commercially distributed (Joos, 1997).

2.IX: BENEFITS OF TRANSFORMATION

Despite the problems associated with plant transformation, the technique boasts many features that make it an effective means of producing plants with specific phenotypic traits. Plant transformation requires less time to obtain desired phenotypic traits than classical breeding methods. Plant transformation can also deal with single gene effects rather than multiple gene effects often seen in classical breeding.

Classical breeding methods require great amounts of time and patience before a plant line with the desired phenotype is produced. Many crops, including canola, require a minimum 4-5 month growing period before seed can be harvested. If a series of back crosses, reciprocal crosses, and self-pollinations are required to obtain the desired phenotype, it can require many years of plant breeding before the new plant line is achieved. Plant transformation is a much faster method of developing a new plant line. Usually, only 6-7 months is required to transform plant tissue with foreign DNA and collect seed from the regenerated plant (Radke et al., 1988). So, while classical breeding often requires years to produce a plant line with a specific phenotype, plant transformation can produce plants with particular phenotypes in only a matter of months.

While transformation deals with individual genes, classical breeding deals with phenotypes, not the genes themselves. As many of the economically important traits are controlled by a large number of genes, or by gene families, classical breeding tends to deal with 'minor' genes which, individually, impart a small effect. In transformation, genes which, individually, impart a major effect are of greatest interest. The use of plant transformation allows the insertion or modification of single genes in the plant genome. In this way, the effect of single genes on plant phenotypes can be determined, which is advantageous both scientifically and economically. Individual genes that impart an economically valuable trait to a plant can be inserted into existing plant lines to improve the economic value (Kramer and Redenbaugh, 1994). Transformation is likely to be most beneficial when dealing with traits controlled by a small number of genes, such as disease resistance or color traits, but may not prove to be as beneficial when examining traits controlled by a large number of genes, such as growth regulation.

Another advantage of transformation is the ability to override incompatability. Classical breeding can only be applied when the species being crossed are compatible. However, transformation can allow a gene from one species to be expressed in an incompatible species. For example, the gene encoding the anticoagulant protein gene, hirudin, found originally in leeches, can be expressed in canola (Moloney et al., 1993). Expression of genes found in other taxa cannot be achieved using classical breeding techniques.

2.X: PROBLEMS WITH ANTISENSE

Just as plant transformation is not a perfect system for generating new plant lines, antisense RNA is not a perfect method for inhibiting gene expression. The biggest problem with antisense technology is its unpredictability. When an antisense gene in transferred to a plant cell, the site of insertion into the genome cannot be predicted, the extent to which the antisense gene will be expressed is unknown and the overall effect of the antisense RNA on the expression of the target gene can only be speculated.

At this point in time, the site at which antisense genes insert into the plant genome is uncontrolled. If the gene is inserted into an actively transcribed section of DNA, one would expect the gene to be vigorously expressed. However, if the antisense gene is inserted into an inactive DNA segment, the gene may not be transcribed at all. Experiments have shown that levels of antisense and sense RNA can vary among individuals transformed with the same antisense construct (Flachmann and Kuhlbrandt, 1995). It may be possible that a different insertion site in each individual may be responsible for variations in antisense expression. While the site of insertion may play an important role in the expression of an inserted antisense gene, it is beyond the abilities of the researcher to control where the antisense gene inserts in the plant genome.

It is also difficult to predict the effect of the antisense RNA on the target gene. Stockhaus found that transformed plants without reduced target RNA had a distinct antisense phenotype but expressed no detectable antisense RNA (Stockhaus et al., 1990). However, Flachmann and Kuhlbrandt found that transgenic plants with very low levels of target RNA and detectable levels of antisense RNA failed to show the expected antisense phenotype (Flachmann and Kuhlbrandt, 1995). It is very difficult to predict how an antisense gene, once inserted into the plant genome, will affect the expression of its target gene.

2.XI: BENEFITS OF ANTISENSE

Despite its drawbacks, antisense technology boasts a number of exciting features, including the ability to study single gene effects. Antisense technology also shows potential for developing crops with high economic value.

One of the positive features of antisense technology is the ability to target single genes for study. As long as the DNA sequence of a particular gene is known, that gene can be antisensed to repress expression of the target gene. Inhibition of gene activity is a recognized method of studying gene function during normal plant growth. As well, the expression of a target gene can be inhibited without affecting other genes (Kohno-Murase et al., 1995).

Antisense technology also shows some promising economic benefits. By using antisense to alter the accumulation of the cruciferin seed storage protein in *Brassica napus*, it has been possible to improve seed storage protein quality (Kohno-Murase et al, 1995). A more renowned example of the economic benefits of antisense is the development of the FLAV'R SAV'R tomato. Antisense technology was used to suppress the activity of the polygalacturonase enzyme, which is responsible for fruit softening (Kramer and Redenbaugh, 1994). With the enzyme activity suppressed, the tomato retains a firmer texture for an extended period of time, making it quite appealing to shippers. The FLAV'R SAV'R tomato has received FDA approval in the United States and promises to be very successful in the marketplace.

Antisense RNA has repeatedly proven to be effective in reducing target gene expression. The chalcone synthase enzyme in petunia (van der Krol et al., 1990) and the acid invertase enzyme in tomato (Ohyama et al., 1995) have both been successfully suppressed by antisense constructs incorporated into the respective plant genomes.

2.XII: CONCLUSION

In order to design a system that will reduce the occurrence of green seed in canola crops, it is important to understand the processes that cause the seed to become green as well as the processes responsible for seed degreening. Chlorophyll and Cab protein biosynthesis, the development of the photosystems and the processes of photosynthesis are all important events that allow the seed to become a green carbon sink during seed development. The action of enzymatic and non-enzymatic chlorophyll degradation systems allow the seed to degreen. However, the occurrence of a sub-lethal frost not only inhibits the normal pigment degradation systems, it can also induce renewed pigment synthesis. The inhibition of pigment degradation and renewed pigment synthesis culminate in the production of mature green seed.

One may attempt to alleviate the green seed problem by hastening chlorophyll degradation in the seed, or inhibiting chlorophyll synthesis. However, the most promising approach to alleviating the green seed problem may be reducing accumulation of chlorophyll in the seed. This particular approach to solving the green seed problem would make use of a number of tissue culture and molecular techniques, including *Agrobacterium tumefaciens*-mediated plant transformation and antisense RNA technology. *Agrobacterium*-mediated plant transformation makes use of the natural ability of the soil bacteria to transfer part of its DNA to an infected plant cell, where that DNA segment is incorporated into the plant genome. Antisense technology is based on the interaction between RNA from an inverted copy of a particular gene and RNA from the gene in its original orientation. The interaction between RNAs reduces the expression of the target gene.

Despite problems associated with both plant transformation and antisense technology, both techniques appear to be effective in accomplishing their respective tasks of delivering foreign genes into a plant genome and reducing target gene expression. Antisensing the type I cab gene of Photosystem II in *Brassica napus* cv. Westar, using methods of plant transformation and antisense technology, should prove to be an effective means of reducing the accumulation of Cab polypeptides in seeds of *Brassica napus*. It is expected that the consequence of Cab reduction will be low seed chlorophyll levels, and a decrease in the occurrence of mature green seed in canola crops.

3: MATERIALS AND METHODS

3.I: GENERATION AND IDENTIFICATION OF TRANSGENICS

3.I.a: Construction of plasmid

The pNABI T-DNA construct (Fig. 2) used for transformation of Westar cotyledons was built by Laurian Robert of Agriculture Canada in Ottawa. The promoter region of the *Brassica napus* napin seed storage gene, gNA, was fused to the 5' end of a cDNA of a *Brassica napus* type I cab gene of Photosystem II in antisense orientation. The 3' end of the antisense fragment was ligated to the 3' terminating sequence of the napin gene. An additional fragment containing a Gus/nptII chimeric segment driven by a double 35S cauliflower mosaic virus promoter was fused to the 3' end of the napin terminator. An avian mosaic virus (AMV) translational enhancer sequence was added to guarantee efficient translation of the message. The Gus/nptII fragment was capped at the 3' end by the nopaline synthase terminator, TER. The antisense construct was positioned in the T-DNA with the napin promoter near the right border and the NER terminator near the left border.

The pNABI construct was harbored in a modified BIN 19 vector (Bevan, 1980), BINSYN. The BINSYN vector, engineered by R. Datla of the Plant Biotechnology Institute in Saskatoon, Saskatchewan, contains synthetic borders which replace the borders found in BIN 19. As in BIN 19, BINSYN houses a gene which confers bacterial kanamycin resistance. The pNABI construct was inserted into BINSYN at a KpN restriction site.

The *Agrobacterium tumefaciens* strain used for cocultivation was GV3 101 pmP 90, obtained from Jas Singh in Ottawa. GV3 101 pMP 90 contains chromosomal genes which confer both rifampicin and gentamycin resistance.

3.I.b: Cocultivation

Brassica napus cv. Westar was chosen as the experimental system for this study due its relatively high amenity for transformation. Seed *of Brassica napus* cv. Westar were surface sterilized in 3% NaOCl for 13 minutes, rinsed several times in sterile water, rinsed again in 90% ethanol and allowed to air dry. Using aseptic technique, the sterile seed were then transferred onto small (60 x 15 mm) petri plates containing solid Murashige Skoog (MS) medium at a density of 30 seed per plate. The plates were placed in sterile inverted Magenta boxes (Magenta Corp. Chicago, Ill.) and the seed samples were allowed to germinate for 5 days under light conditions of 100 $\mu E/m^2/s$ in a 16 hour photoperiod at 25° C. The light source included a combination of warm white General Electric and cool white Silvania fluorescent bulbs.

Agrobacterium containing the pNAB1 construct were grown overnight at 28° C, in 10 mls of Luria Bertani (LB) media (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.5% w/v NaCl, pH 7.5) containing 120 µg/ml rifampicin, 100 µg/ml gentamycin and 50 µg/ml kanamycin. Cells were centrifuged for 5 minutes at 2700 rpm in a clinical centrifuge and the resulting bacterial pellet was resuspended in fresh LB without antibiotics to an OD_{600} of 0.6.

In a flow bench, using aspetic technique, the cotyledons were excised from the hypocotyls of 5 day old Westar seedlings in such a way that the cotyledon retained approximately 3 mm of petiole. The base of the petiole was dipped in the *Agrobacterium* suspension before being plated onto solid MS medium supplemented with 1.25% benzyladenine,. The cotyledons and *Agrobacterium* were cocultivated for two days in the same light and temperature conditions used for germination. The cotyledons were then transferred to solid MS medium supplemented with benzyladenine (1.25%) and 0.5 mg/ml carbenicillin for one week. The carbenicillin acts to eliminate growth of *Agrobacterium*.

After one week the cotyledons were moved to selection medium (MS, 1.25% benzyladenine, 0.5 mg/ml carbenicillin, 15 µg/ml kanamycin). Kanamycin in the medium bleaches the leaves of any non-transformed shoots that may emerge from the developing callus. The cotyledons were sub-cultured every three weeks onto fresh selection medium until callus and shoot development was evident. During this time on selection medium, the light intensity was reduced to 60-80 $\mu E/m^2/s$, the photoperiod was maintained at 16 hours and the temperature was 24° C.

Once green shoots reached 2-5 cm in length, the entire callus was aseptically transferred to rooting media in a sterile Magenta box. Rooting medium contained MS medium supplemented with 2 mg/l of the auxin, indole butyric acid, and 0.5 mg/ml carbenicillin. Shoots were maintained on rooting medium for several weeks until roots became visible. Individual plantlets were then separated from the callus and transferred to 7 cm pots filled with sterile soil (40% vermiculite, 40% peat moss, 20% sand and trace amounts of 14-14-14 fertilizer, dolomite, super phosphate, Fe chelate, and trace elements). The putatively transformed plantlets were grown in a misting chamber under conditions of 60 $\mu E/m^2/s$, 25°C, 16 hour photoperiod and 100% relative humidity. Over a period of 6-8 weeks, the plantlets were hardened off by slowly decreasing the humidity of the misting chamber from 100% to greenhouse conditions.

Plantlets that survived selection on kanamycin were re-potted into 15 cm pots and transferred to the greenhouse. Tissue culture control (Control) plantlets regenerated from non-cocultivated cotyledons were also transferred to the greenhouse. Greenhouse conditions included a 16 hour photoperiod and 24°/20° C diurnal temperatures.

3.I.c: Gus assays

Plantlets were tested for the expression of the Gus gene located in the pNABI construct. Histochemical Gus assays (Jefferson, 1985) were performed on leaf tissue from each plantlet derived from tissue culture. Individual leaf discs were placed in 1.5 ml microfuge tubes which were then filled with 300-500 µl X-gluc substrate (0.05% w/v 5-bromo-4-chloro-3-indolyl glucuronide (Sigma Chemical Company) and 0.5% v/v dimethyl formamide in 50 mM NaH_2PO_4, pH 7.0). The leaf discs were vacuum infiltrated with the X-gluc substrate for 30 minutes in a glass desiccator attached to a vacuum line. Following vacuum infiltration, the leaf discs were incubated in X-gluc overnight at 37°C. Chlorophyll was cleared from the leaf discs by incubation in 1.5% NaOCl for one hour. Clearing chlorophyll from the leaf discs allowed easier visualization of the blue precipitate produced by the Gus enzyme in the presence of X-gluc. Plantlets that survived kanamycin selection during shoot formation and tested positive for Gus activity were considered putative transgenics.

3.I.d: DNA extraction

DNA to be used as PCR template was extracted from leaf tissue using modified methods of Dellaporta et al. (1983). The only modification made to the protocol was the addition of a phenol/chloroform extraction performed prior to DNA precipitation with isopropanol.

3.I.e: PCR with nptII primers

The putative transgenics were tested for the presence of the nptII marker gene by PCR. DNA to be used as PCR template was extracted from leaf tissue of Westar and Control plants, as well as DII and DIII plants in the T2 generation using slightly modified methods of Dellaporta (1983). The only modification made to the Dellaporta protocol was the addition of a phenol/chloroform extraction performed prior to DNA precipitation with isopropanol. Each PCR reaction was carried out in 50 µl volumes containing 1 mM $MgCl_2$, 1X PCR buffer (Gibco BRL), 200 mM each of dNTPs, 1 µg of each nptII primer, 1 µg of DNA template and 2 units of Taq polymerase (Gibco BRL). Initial denaturation conditions during PCR was 94° C for 4 minutes, followed by 35 cycles of 94°C denaturation for 1 minute, annealing at 59° C for 1 minute and extension at 72° C for 2 minutes. The PCR reaction was performed using the PHC 2 Dri-Block ^{R} Techne Corporation thermal cycler. NptII primers, supplied by Jas Singh of Ottawa, were used to amplify a 0.7 Kb fragment between the 223-900 nucleotides in the nptII gene. The nptII primers encode 21 bp fragments between the 201-222 nucleotides and the 900-879 sequences in the nptII gene itself (Beck et al., 1982). The nptII primer sequences are listed below.

GAGGCTATTCGGCTATGACTG (positions 201-222 in nptII gene)
ATCGGGAGCGGCGATACCGTA (positions 900-879 in nptII gene)

Those plantlets identified by PCR as carrying the nptII gene were considered transgenic and became our primary transgenics, the T0 generation.

PCR products were detected after electrophoretic separation on 0.8% agarose/EtBr gels using TAE (0.05% w/v Tris, 0.01% v/v acetic acid and 0.02% v/v .5M EDTA, pH 8.0) as running buffer. The small 30 ml agarose/EtBr gels used to analyze PCR products were run in an MLB-06 model gel box (Tyler Research Instruments, Edmonton, Alberta, Canada).

3.I.f: Obtaining the T1 and T2 generation

Primary transgenics were self pollinated to obtain the T1 generation. T1 seed were germinated on soil (as described in 3.I.b) in the greenhouse. Leaf tissue from the T1 seedlings was used in histochemical Gus assays to determine whether the pNABI construct was inherited from the primary transgenics to the offspring.

T1 seedlings testing positive for Gus activity were maintained under greenhouse conditions in 15 cm pots at a density of 2 plants per pot. During flowering of the T1 plants, large buds (5-6 mm in length) were emasculated, hand pollinated and tagged with the date of pollination. All unpollinated buds and flowers were removed from the plant.

Siliques were removed from the T1 plants at 15, 18, 23, 28, 33, 38 and 43 days after the pollination date (DPA) and the T2 seed was collected. T2 seed collected from sister plants were pooled. Subsamples of 15-30 seed were used to determine the seed moisture content and average dry weight of each seed sample. The seed not used for dry weight and seed moisture determinations were frozen in liquid nitrogen and stored at -80° C. These frozen seed samples were used for chlorophyll determination and protein extraction at a later date.

A minimum of 4 separate harvests were conducted for each of the 7 different DPA periods for each of the following plant lines: Westar, tissue culture control (Control), and 2 transgenic lines, DII and DIII. A total of 112 seed samples were collected for all seed lines.

3.I.g: Gus assays to establish homozygosity

In order to determine the levels of homozygosity obtained in T2 transgenic plants, Gus assays were performed to determine the inheritance of the pNABI construct. Small leaves, approximately 1 cm in diameter were excised from Westar, Control, DII and DIII plants. Leaves from 24 DII and 24 DIII plants were subjected to histochemical Gus assays as described earlier. Leaves from the 10 Westar and 10 Control plants were used as negative controls. Leaf tissue from a *Brassica napus* transformed with a Gus-containing FV4 based vector, provided by Jas

Singh of Ottawa, was used as a positive control. Transgenic lines heterozygous for the pNABI construct were expected to produce several Gus-negative plants while plant lines homozygous for the pNABI construct were expected to produce only Gus-positive plants.

3.I.h: PCR with Gus primers

PCR was used to confirm the presence of the pNABI construct in the T2 generation of the DII and DIII transgenic lines. Gus primers were used to amplify a 1.2 Kb fragment between the 420-1570 nucleotides in the Gus gene. The primers encode 21 bp fragments between the 400-420 nucleotides and the 1599-1579 nucleotides (Jefferson et al., 1987) in the Gus gene. The sequences for the Gus primers are listed below.

GGTGGGAAAGCGCGTTACAAG (positions 400-420 in the Gus gene)
GTTTACGCGTTGCTTCCGCCA (positions 1599-1579 in the Gus gene)

3.I.i: Southern blots

DNA to be analyzed by Southern blotting was extracted from T2 leaf tissue using modified methods of Dellaporta et al. (1983). DNA was extracted from Westar, Control, DII and DIII leaf tissue. DNA (15 µg) from each of the 4 plant lines was digested with Pst I. The resulting DNA fragments were separated by electrophoresis on large 100 ml 0.8% agarose/EtBr gels using the HG-20 agarose gel box (Tyler Research Instruments, Edmonton, Alberta). The gel was then depurinated for 10 minutes in 250 mM HCl, incubated for one hour in denaturing solution (0.5 N NaOH, 1.5 M NaCl) followed by a one hour incubation in neutralizing solution (1.0 M Tris-HCl, pH 8.0, 1.5 M NaCl). The DNA was then transferred overnight from the agarose gel to a nylon membrane (Zeta-Probe blotting membrane, Bio-Rad Laboratories, Hercules, California) via capillary action. DNA was fixed to the membrane by baking at 80° C under vacuum for 1 hour. The blotted membrane was incubated in prehybridization solution (50% formamide, 0.12 M Na_2 HPO_4 , pH 7.2, 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA) at 43° C for 5 minutes. The membrane was then sealed in a plastic bag with the ^{32}P labeled probe diluted to 50 ng/ml in fresh prehybridization buffer. A 2.75 Kb Bam HI fragment of the Gus/nptII coding region in the pNABI construct was randomly labeled with ^{32}P and used to probe the nylon membranes. The membrane was incubated in the probe at 43°C overnight. After incubation, the membrane was rinsed briefly in 2X SSC (0.3 M NaCl, 30 mM Na citrate, pH 7.0) followed by an initial washing in 2X SSC, 0.1% SDS for 15 minutes at room temperature. The membrane was then washed in 0.5X SSC, 0.1% SDS for 15 minutes at room temperature. Finally, the membrane was washed in 0.1X SSC, 0.1% SDS at 65° C for 10 minutes. The membrane was then sealed in a plastic bag and exposed to x-ray film (Fuji) for 4 days at -80° C.

3.II: CALCULATION OF MEAN DRY WEIGHT PER SEED AND PERCENT SEED MOISTURE

Subsamples of 20-30 seed were obtained at 7 different DPA from each harvested Westar, Control and T2 transgenic seed sample. Each subsample was weighed, then dried overnight at 80° C in a vacuum oven. The seed subsamples were weighed again after drying. To determine the averaged dry weight/seed, the following calculation was performed for each subsample:

Weight Y / number of seed in subsample = average dry weight /seed

where Weight Y is the total dry weight of the seed subsample.

To determine the percent moisture content of the seed sample, the following calculation was performed for each seed subsample:

([Weight X] - [Weight Y]) / Weight X x 100 = percent seed moisture of sample

where Weight X is the total fresh weight of the seed subsample. Mean dry weight per seed and percent seed moisture were measured using a minimum of 3 subsamples of seed.

3.III: CHLOROPHYLL DETERMINATION

Chlorophyll content was measured for seed from Westar, Control, and T2 seed from the transgenic lines during seed development. Chlorophyll contents of 4 different seed samples were determined in less than 1 ½ hours using the following method. The protocol used for chlorophyll extraction was modified from Vernon (1960). Seed were counted and were ground to a fine powder under liquid nitrogen in a chilled mortar and pestle kept on ice. Seed was ground again in 200 µl of 80% acetone and the slurry was transferred to a 2 ml screw cap microfuge tube. Tubes were microcentrifuged in the dark at 4°C for 10 minutes at 13,000 rpm. The supernatant was transferred to a flask kept on ice in the dark. The pellet was resuspended in 80% acetone, microcentrifuged as above and the supernatants combined. This process was repeated until the pellet was white. The combined supernatants were centrifuged at 4°C for 5 minutes at 13,000 rpm, transferred to a volumetric flask and the final volume was brought to 5 ml or 10 ml with 80% acetone. Absorbance of the supernatant was read at A_{649} and A_{665} in a spectrophotometer (Cary 210 dual beam spectrophotometer, Varian Techtron LTD, Australia). The absorbance readings of 2 aliquots of each supernatant were taken and averaged. Chlorophyll content for each seed sample was determined by the following equations (Vernon, 1960).

Chl a (µg/ml) = 11.63(A665) - 2.39(A649)

Chl b (µg/ml) = 20.11 (A649) - 5.18 (A665)

Total Chl (µg/ml) = 6.45 (A665) + 17.72 (A649)

The information was then used to determine chlorophyll content/seed and chlorophyll content/gram dry seed weight for each seed sample.

3.IV: PROTEIN EXTRACTION AND ANALYSIS

3.IV.a: Protein extraction

In order to remove the lipids that distort protein bands during SDS-PAGE, samples of 20-30 seed were ground to a fine powder in liquid nitrogen in a chilled mortal and pestle kept on ice, then reground in 80% acetone. The homogenate was transferred to a 2 ml screw cap microfuge tube and microcentrifuged at 4° C for 10 minutes at 13,000 rpm. The supernatant was discarded and the pellet resuspended again in 80% acetone. The homogenate was microcentrifuged as above and the supernatant discarded. This process was repeated once with 80% acetone and then with 100% acetone until the pellet was white The pellet was then air dried, followed by resuspension in 800-1000 μl of SDS reducing buffer (10% glycerol, 5% fresh β-mercaptoethanol, 2% SDS, and 0.05% bromophenol blue in 62.5 mM Tris-HCl, pH 8.0 [all v/v]).

The resuspended pellet was incubated in a 95° C water bath for 15 minutes with occasional vigorous vortexing. The suspension was then microcentrifuged at room temperature for 10 minutes at 13,000 rpm and the supernatant transferred to sterile microfuge tubes in aliquots of 200 μl. SDS-solubilized protein samples were then frozen in liquid nitrogen and stored at -20° C.

3.IV.b: TCA protein precipitation and quantification

SDS-soluble protein samples were precipitated in 2 ml screw cap microfuge tubes with 2 volumes of 10% trichloroacetic acid at room temperature for 15 minutes followed by microcentrifugation at 13,000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet allowed to air dry. Once dry, the pellet was resuspended in 200 μl 0.1 N NaOH with heating.

To determine the concentration of precipitated protein samples, 6 μl aliquots of NaOH protein suspensions were diluted 1/100 with dd H_20. The remaining NaOH protein suspension was frozen in liquid nitrogen and stored at - 20° C until electrophoretic analysis. 50 μl aliquots of the diluted protein solution was added to 500 μl of BCA protein assay working. The protein/BCA samples were briefly vortexed, then heated in a 60° C water bath for 30 minutes. The protein/BCA samples were left to cool at room temperature for 5 minutes before measuring the spectrophotometric absorbance at A_{562}. Spectophotometric readings were compared to a BSA standard curve in order to determine the protein concentration for each tested protein sample. The 1/100 dilutions were performed in duplicate for each sample and each dilution was assayed in duplicate to ensure accurate determination of protein concentration. The protein concentration for each of the 4 examined plant lines was calculated from 6 separate protein determinations.

3.IV.c: SDS-PAGE

All polyacrylamide gels used for this research were 16 cm in length and 1.5 mm thick. Polyacrylamide gels were assembled using a 12.5% polyacrylamide separating gel and a 4% polyacrylamide stacking gel. The acrylamide to bis-acrylamide ratio used for all SDS-PAGE gels was 30%/0.8%. Acrylamide was polymerized with final concentrations of 0.1% TEMED and 0.2% ammonium persulfate. Gels were run using the Laemmli discontinuous buffer system (Laemmli, 1970). Gels comparing Cab protein accumulation among the 4 examined plant lines at a single DPA were loaded on the basis of equal total protein, according to protein concentrations determined by BCA protein assays or were loaded on a per seed basis. Gels comparing Cab protein accumulation in a single plant line over a time course were loaded on a per seed basis. Prior to loading, all NaOH protein suspension samples were combined with at least one equal volume of SDS reducing buffer which also acted as tracking dye. Samples were then boiled for 5 minutes, cooled on ice and microcentrifuged at 13,000 rpm for 10 minutes at 4°C before loading on the gel.

Gels were run for 5-6 hours in a large gel apparatus (Bio Rad Protean TM II) at 30 mA constant current. Polypeptides were then electrophoretically transferred from polyacrylamide gels to nitrocellulose membrane (BA-S 83-o.2 mm pore, Schleicher and Schuell, Keene, NA) for 3 hours at 70 volts using a TE series Transphor Electrophoresis unit (Hoefer Scientific Instruments, San Francisco, California) filled with transfer buffer [10 mM $NaHCO_3$, 3 mM Na_2CO_3, and 20% methanol (Dunn, 1986)]. Cold water running through a glass cooling core was used to maintain the temperature of the transfer buffer below 25°C.

The efficiency of electrophoretic transfer of polypeptides from the gel to nitrocellulose was checked by staining gels overnight in a Coomassie blue solution (0.1% Coomassie blue R-250 in a 40% methanol, 10% acetic acid solution). Untransferred polypeptides were visible in the gel after destaining in a 40% methanol,10% acetic acid solution for 5 hours.

3.IV.d: Ponceau staining and western blotting

Blotted nitrocellulose membranes were stained for 30 seconds with Ponceau Red protein stain (0.5% w/v Ponceau S, 1% acetic acid) and destained briefly with distilled water in order to visualize polypeptides transferred from the gels to nitrocellulose. Equal loading of proteins was also assessed visually on Ponceau stained membranes. Membranes were destained for 30 minutes or overnight in distilled water at room temperature to remove the Ponceau stain.

Upon removal of Ponceau stain, membranes were incubated at room temperature for 1 hour in blocker [3% w/v Telostean (Sigma Chemical Company), in 1X PBS (1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.4)]. Following blocking, membranes were submerged in 20 ml of primary antibody (α-CPIa antiserum diluted 1:400 in blocker containing 0.001% NaN_3). The α–CPIa antibody identifies Cab polypeptides from both the LHCII and LHCI antenna complexes (White and Green, 1987). After a 2 hour incubation in primary antibody, membranes were washed 3 times with 0.05% Tween 20 in 1X PBS for 5 minutes, followed by a fourth wash in 1X PBS for 5 minutes. Following the series of PBS washes, the membranes were incubated for 2 hours in secondary antibody (1:3000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma Chemical Company) in blocker. After incubation with secondary antibody, the membranes were again washed 3 times with 0.05% Tween in 1X PBS, each for 10 minutes followed by a single 10 minutes wash in 1x PBS. The membranes were then equilibrated in 250 mM Tris-HCl (pH 8.0) for 5 minutes followed by color development in Fast Red reaction solution (0.2% w/v Fast Red, 0.1% w/v Napthol AS-MX Phosphate (Sigma) in 50 mM Tris-HCl (pH 8.0) for 50 minutes (White and Green, 1987a).

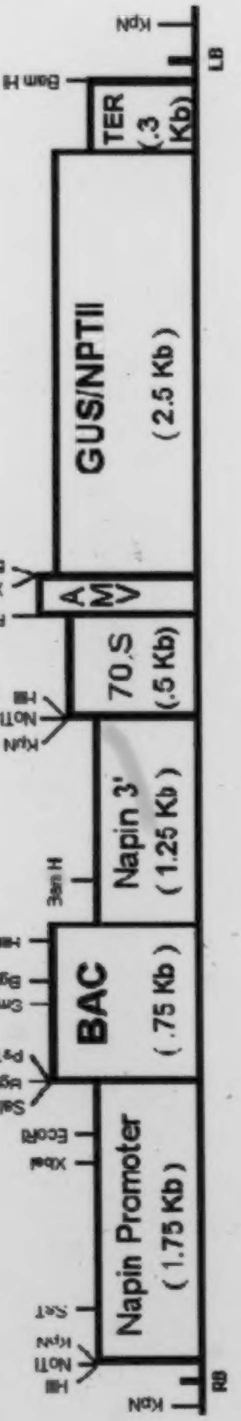
3.IV.e: Densitometry of westen blots

Western blots to be used for densitometric analysis were scanned using the Bio Rad Model GS-670 Imaging Densitometer (Bio Rad, Hercules, CA). Once the western blots were scanned, the images were subjected to densitometric analysis using Molecular Analyst^R software from Bio Rad (Bio Rad, Hercules, Ca). The volume data generated by Molecular Analyst^R was calculated using global background. All data points were within the acceptable linear range.

3.V: STATISTICAL ANALYSIS

All computations were performed using SAS^R (Statistical Analysis System, Sas Institute, Cary, NC). To determine whether or not chlorophyll and Cab levels were correlated, the Pearson correlation coefficient was calculated for each individual plant line and for all 4 plant lines together. To determine whether or not chlorophyll levels among the plant lines were significantly different during seed development, a general linear model was used to determine the least significant difference using a confidence level of 0.1. Least significant difference was also calculated to compare Cab accumulation among the 4 plant lines during seed development and the mean chlorophyll content in the seed of Westar, Control, DII and DIII. Significant differences were calculated with the effects of several variables on chlorophyll and Cab protein accumulation taken into consideration. The effects of replicate, plant line, DPA, replicate by plant line, replicate by day and plant line by day on chlorophyll and Cab protein accumulation were examined.

FIG. 2: The pNABI antisense cab construct. Right border, (RB); Napin seed storage protein (gNa) promoter for driving transcription of antisense gene; BAC reverse oriented cab gene; Napin 3' terminator; 70S (double 35S) constitutive promoter from cauliflower mosaic virus for driving transcription of detectable/selectable markers; AMV avian mosaic virus translational enhancer sequence; Gus gene for β-glucuronidase detectable marker; nptII gene for neomycin phosphotransferase selectable marker; TER 3' terminator; Left border (LB). Known restriction sites are shown. Total length of construct was approximately 7 Kb.


KpN
HIII
NoTI
KpN
RB
SsT
Napin Promoter
(1.75 Kb)
XbaI
EcoRI
Sal I
BglII
PsTI
BAC
(.75 Kb)
Sma I
BglII
HIII
Bam H
Napin 3'
(1.25 Kb)
KpN
NoTI
HIII
70 S
(.5 Kb)
BglII
XbaI
Bam HI
AMV
GUS/NPTII
(2.5 Kb)
TER
(.3
Kb)
Bam HI
LB
KpN

4: RESULTS

4.I: PRIMARY TRANSGENICS

4.I.a: Obtaining putative transgenics

Transformation of approximately 4000 Westar cotyledons with the pNABI antisense construct produced four green shoots that survived selection on kanamycin. These green shoots were considered putative transgenics. Survival of shoots on kanamycin supplemented medium suggested the presence of the nptII gene from the pNABI construct in the plant genome.

4.I.b: Gus assays to screen for transgenics

The putative transgenic shoots were screened for the presence of the pNABI construct by performing histochemical Gus assays on leaf samples obtained from the shoots (Fig. 3). Of the five shoots tested, three displayed positive Gus activity. These three shoots were subsequently referred to as CII, DII and DIII.

4.I.c: PCR to verify primary transgenics

To confirm the transgenic nature of the shoots showing positive Gus activity, PCR was performed to identify the presence of the nptII gene within the putatively transgenic plant genomes. NptII primers were successfully used to amplify a 0.7 Kb fragment from DNA extracted from CII, DII and DIII leaf tissue (Fig. 4). Identification of the 0.7 Kb fragment suggested successful incorporation of the pNABI construct into the genomes of plants CII, DII and DIII. These primary transgenic plants were referred to as the T0 generation.

4.I.d: Gus assays to identify successful inheritance of the pNABI construct in the T1 generation

The primary transgenic plants, CII, DII and DIII, were selfed to produce the T1 generation. In order to ensure that further experimentation would only involve transgenic plants, Gus assays were performed on T1 seedlings to identify plants containing the pNABI construct. The T1 generations of DII and DIII contained both Gus positive and Gus negative individuals (data not shown). However, the T1 generation of CII contained only Gus negative plants. Therefore, only DII and DIII were used for further study.

4.II: T2 TRANSGENIC GENERATION

4.II.a: PCR to verify inheritance of pNABI construct in the T2 generation

After selfing primary transgenics to obtain the T1 generation, the Gus positive members of the T1 generation were selfed again to produce the T2 generation. In order to ensure that the pNABI construct was inherited by the T2 generation, PCR was performed to confirm the presence of the Gus gene from the pNABI construct in the genomes of the DII and DIII plants. A 1.2 Kb fragment was identified in both DII and DIII plants but not in the Westar nor the Control plants (Fig. 5). The presence of the 1.2 Kb fragment suggests successful inheritance of the pNABI construct from the T0 to the T2 generation.

4.II.b: Southern blot analysis to determine copy number

In order to identify the number of copies of the pNABI construct that were inserted into the transgenic plant genomes, southern blot analysis was performed. Plant genomic DNA was restricted with the Pst I restriction enzyme and the resulting cleaved DNA subjected to southern blot analysis. The Southern blot was probed with a ^{32}P labelled 2.7 Kb Gus/nptII fragment of the pNABI construct. The results revealed no hybridizable fragments in the Westar and Control lanes (Fig. 6). In both the DII and DIII lanes, two bands were detected. The band sizes were identical in both transgenic lanes. In order to determine whether or not the probe contained a Pst I restriction site, 2 µg of the 2.7 Kb Gus/nptII fragment were restricted with Pst I and seperated on an agarose gel. The results of the probe restriction indicate that the probe was cleaved by the Pst I enzyme (Fig. 7). Since the probe was cleaved by the Pst I restriction enzyme, the Gus/nptII fragment of the pNABI construct incorporated into the plant genome would be cleaved during the genomic DNA restriction. This cleavage of the 2.7 Kb Gus/nptII fragment accounts for the presence of two bands, rather than a single band, in the Southern blot of the transgenic plant lines. The presence of two bands in the Pst I restricted Southern blot of DII and DIII DNA indicate the presence of a single copy of the pNABI construct in the transgenic plant genomes.

4.II.c: Gus assays to determine homozygosity of T2 generations of DII and DIII

In order to determine the level of homozygosity of the pNABI construct obtained in the T2 generation, histochemical Gus assays were performed on leaf tissue samples from the T2 generations of DII, DIII, and from Westar and Control (Table 1). Of 24 DIII plants assayed, all tested positive for Gus activity. Of 24 DII plants assayed for Gus activity, 21 plants tested positive while three plants tested negative. Of the 10 Westar plants and 10 Control plants assayed for

Gus activity, all tested negative. These results suggest that DIII may be homozygous for the pNABI construct. However, the ratio of Gus positive/Gus negative DII plants suggests that the pNABI construct in the DII genome is not homozygous.

4.III: CHANGES IN CHLOROPHYLL LEVELS

4.III.a: Peaks in total chlorophyll accumulation (µg/seed)

The time of maximum chlorophyll accumulation was very similar for all four plant lines. While maximum chlorophyll accumulation for Control, DII and DIII was measured at 28 DPA, maximum chlorophyll accumulation was measured in Westar shortly thereafter, at 33 DPA. Although maximum chlorophyll accumulation in Westar was detected 5 days after the other plant lines (Table 2), maximum chlorophyll levels for Westar, Control and DII were within a standard error of one another (Fig. 8). Chlorophyll peaks for Westar and DIII were just outside a standard error of one another, with Westar containing higher chlorophyll levels relative to DIII, particularly at 33 DPA. Overall, there was a clear trend of higher total chlorophyll for Westar relative to the other plant lines, except at 43 DPA. DII appeared to exhibit higher total chlorophyll levels than DIII. The greatest differences in total chlorophyll were seen among the plant lines after 28 - 33 DPA. It appears then that maximum chlorophyll levels in DIII were noticeably lower than maximum chlorophyll levels found in Westar.

4.III.b: Rate of chlorophyll loss

Examination of total chlorophyll levels (µg/seed) (Fig. 8) after 28 DPA revealed that rapid chlorophyll loss in the transgenics appeared to occur between 28 and 33 DPA and again between 38 and 43 DPA. In comparison, losses in Westar were linear between 33 and 43 DPA. However, despite the apparent differences in rates of chlorophyll loss between transgenics and Westar, examination of the standard error for the four plant lines reveals that these differences in rates of chlorophyll loss were within a standard error. So although the rates of chlorophyll loss appear to differ between Westar and the transgenics, the size of the standard error negates such a conclusion.

4.IV: CHLOROPHYLL

4.IV.a: Chlorophyll content in the developing seed of the T2 generation

The chlorophyll content in the T2 seed of the two transgenic lines were compared to Westar and Control lines over the 15 DPA - 43 DPA time course of seed development. Chlorophyll contents were calculated for a variety of categories, including chlorophyll/seed, chlorophyll/gram dry weight and chlorophyll/gram protein. Chlorophyll a, chlorophyll b, and total chlorophyll levels were determined for the above categories..

4.IV.b: Chlorophyll a (μg/seed)

When comparing chlorophyll a levels on a per seed basis, it was revealed that there was no significant difference between Westar, Control, DII and DIII seed at 15 and 18 DPA (Fig. 9). However, at 23 DPA, DIII appeared to contain noticeably less chlorophyll a than did Westar. At 28 DPA, chlorophyll a in the four plant lines returned to approximately equal levels. At 33 DPA, chlorophyll a levels in DII, DIII and Control dropped significantly while chlorophyll a levels in Westar reached their peak. The greatest difference in chlorophyll a levels was between Westar and DIII, where chlorophyll a levels in Westar exceeded those in DIII by 48%. At 38 DPA, DIII continued to contain less chlorophyll a than did Westar, but chlorophyll a levels had dropped in all plant lines. By 43 DPA, there was no noticeable difference in chlorophyll a levels between Westar, DII and DIII although the Control appeared to have retained more chlorophyll a. These results suggest that the transgenic seed contained reduced chlorophyll a levels relative to Westar at 33 and 38 DPA.

4.IV.c: Chlorophyll b (μg/seed)

The results of chlorophyll b analysis were very similar to those seen in the analysis of chlorophyll a (μg/seed). A comparison of chlorophyll b levels from Westar, Control, DII and DIII seed revealed that there was no difference between the 4 lines during early seed development (15-28 DPA) (Fig. 10). However, by 33 DPA, chlorophyll b levels had begun to drop in Control, DII and DIII while continuing to rise in Westar. These differences in chlorophyll b levels continued to be visible at 38 DPA. By 43 DPA, chlorophyll b levels had fallen such that there was no noticeable difference between Westar, DII and DIII; however, chlorophyll b levels in the Control remained quite high. Again, the transgenics contained reduced chlorophyll b levels relative to Westar at 33 and 38 DPA

4.IV.d: Total chlorophyll (μg/seed)

Examination of total chlorophyll (μg/seed) revealed similar patterns of chlorophyll accumulation and degradation as those patterns seen in chlorophyll a and chlorophyll b analysis. A comparison of total chlorophyll in Westar, Control, DII and DIII seed revealed there was little difference in total chlorophyll accumulation among the 4 plant lines until 33 DPA (Fig. 8). By 33 DPA, chlorophyll levels continued to rise in Westar, but began to drop in the other 3 plant lines. At

33 DPA, Westar contained 48% more total chlorophyll than did DIII and 29% more total chlorophyll than did DII. At 38 DPA, chlorophyll levels started to decline in Westar, but total chlorophyll content continued to exceed DIII by 38%. At 43 DPA, chlorophyll levels in Control seed exceeded levels found in DIII.

4.IV.e: Chlorophyll a (mg/gram dry seed weight)

Chlorophyll was also analyzed and calculated in the 4 plant lines on the basis of dry weight of seed. These calculations addressed any differences that may have been introduced by virtue of varying seed size in the chlorophyll per seed calculations. The results of chlorophyll a (mg/gram dry weight) analysis were very similar to the results of the chlorophyll a (µg/seed) analysis (Fig. 11).

4.IV.f: Chlorophyll b (mg/gram dry seed weight)

Analysis of chlorophyll b on the basis of dry weight of seed revealed very similar results to those seen when chlorophyll b was analyzed on the basis of seed number (Fig. 12).

4.IV.g: Total chlorophyll (mg/gram dry seed weight)

Results of the analysis of the total chlorophyll content of the 4 plant lines, calculated on the basis of dry seed weight, were quite similar to the results of the total chlorophyll (µg)/seed analysis, except for one noticeable difference (Fig. 13). Peak accumulation of total chlorophyll for Westar and the Control was reached at 23 DPA when calculated on a gram dry seed weight basis. However, when calculated on a per seed basis, maximum chlorophyll accumulation was detected for Westar and Control at 33 and 28 DPA respectively. It is important to note that the differences in chlorophyll accumulation (mg/gram dry seed weight) between 23 and 28 DPA do not exceed standard error in Control. Similarly, differences in chlorophyll accumulation (mg/gram dry seed weight) between 23 and 33 DPA do not exceed standard error in Westar. So although peak chlorophyll accumulation is detected at 23 DPA, chlorophyll accumulation is not considerably different than chlorophyll levels found at 28 and 33 DPA, for Control and Westar respectively, when examined in terms of mg total chlorophyll/gram dry seed weight.

4.IV.h: Total chlorophyll (mg/gram total protein)

The amount of total chlorophyll per gram total protein was also examined in each plant line during seed development (Fig. 14). In terms of chlorophyll loss, results were quite similar to the results observed on a per seed and per gram dry weight basis. However, maximum chlorophyll/gram total protein occurred at 23 DPA in all plant lines.

4.IV.i: Statistical analysis

In order to determine whether or not the differences in total chlorophyll accumulation observed among the 4 lines were significant, several computations were performed. Significant differences were calculated for total chlorophyll (µg/seed) data at each of the tested DPA for the 4 lines (Table 3). At the 0.1 confidence level, Westar contained significantly more chlorophyll relative to DIII at 33, 38 and 43 DPA. Chlorophyll content in Westar was also significantly greater than was found in DII at 33 and 38 DPA. The transgenic lines appeared to contain significantly lower chlorophyll levels relative to Westar at 33 and 38 DPA.

Analysis of means (total chlorophyll (µg/seed)) by plant line at a 0.1 confidence interval suggested that the means for Westar, Control and DII do not differ significantly. The means for Control, DII and DIII also did not differ significantly. Total chlorophyll levels in Westar, however, were significantly higher than in DIII. It appears that DIII seed contained significantly less chlorophyll overall during seed development relative to Westar seed.

4.V: CAB PROTEIN

4.V.a: Chlorophyll a/b ratios

Chlorophyll a/b ratios allow us to analyze the relative amounts of Cab proteins in the seed samples (Flachmann and Kuhlbrandt, 1995). Chlorophyll a/b binding proteins are the only proteins that bind chlorophyll b. Therefore, when Cab protein levels decrease, chlorophyll b levels also decrease, resulting in a higher chlorophyll a/b ratio. Chlorophyll a/b ratios were analyzed for seed of Westar, Control and T2 seed of DII and DIII during development (Fig.15). The chlorophyll a/b ratios did not differ among the four plant lines except at 23 DPA and 43 DPA. At 23 DPA, Westar had a much higher chlorophyll a/b ratio than did Control or DIII plants. At 43 DPA, DII and DIII displayed very high chlorophyll a/b ratios compared to the Control or Westar. These results suggest a depletion in Cab protein levels and subsequently chlorophyll b levels at 43 DPA. However, considering the low levels of chlorophyll found in the seed at 43 DPA and the difficulties associated with quantifying very low levels of chlorophyll, the accuracy of the chlorophyll a/b ratio at 43 DPA may be questionable.

4.V.b: Cab protein accumulation during seed development

In order to examine the pattern of Cab protein accumulation during seed development, protein was loaded on SDS-PAGE gels on a per seed basis from each of the 7 developmental stages examined. The resulting western blots showed the developmental sequence of Cab accumulation for Westar, Control, DII and DIII (Fig. 16-19). Densitometry demonstrated that maximum Cab accumulation appeared to occur at 28 DPA in all plant lines (Table 2 and Appendix A). It appears that the presence of the antisense cab gene does not affect the temporal expression of the examined members of the cab gene family.

4.V.c: Representative western blots

Figures 20 and 21 show the results of comparative western blots for 23 and 18 DPA, loaded on the basis of equal total protein and per seed, respectively. A visual assessment of these blots revealed a reduced accumulation in DIII relative to the other 3 lines. Comparative western blots to be used for densitometric analysis were loaded with total protein on an equal per seed basis. Western blots were used to examine Cab accumulation in the four plant lines at 15, 18, 23, 28, 33, 38 and 43 DPA.

4.V.d: Statistical analysis

Significant differences were calculated for Cab protein levels in the 4 plant lines on the basis of OD/mm^2 calculated by densitometric analysis of comparative western blots (loaded on a per seed basis) (Appendix B). When the least significant difference was calculated at a 0.1 confidence interval, significant differences were found in Cab accumulation at 23, 28,33 and 43 DPA (Table 3). At 28 DPA, Westar contained significantly more Cab protein than was present in DIII. Marginal significant differences were found at 33 and 38 DPA, again with levels greater in Westar than in DIII (Table 4). Although the differences between Westar and DIII at 33 and 38 DPA were not calculated to be significant, the differences were considerable and may, nonetheless, be indicative of an antisense effect. Cab levels in Westar did not significantly exceed Cab levels in DII at any examined point during seed development. In the Control, Cab levels were significantly greater than that in DIII at 23, 28, 33 and 43 DPA. At 28 and 43 DPA, Cab levels in the Control were significantly higher than that in DII.

4.V.e: Component Cab bands

Each of the five component bands was examined individually and the point of maximum accumulation for each band was observed. If the point of maximum accumulation for a component band differed from maximum total Cab accumulation, the difference was noted.

In the developmental western for Westar, it was noted that LHCIIb peaked at 33 DPA, while total Cab peaked at 28 DPA. As well, the first of 3 bands comprising LHCI reached maximum accumulation between 28-33 DPA. For Control, developmental densitometry revealed LHCIIb maximum accumulation occurred at 33 DPA, while total Cab peaked at 28 DPA. Densitometry for the developmental western of DII revealed that LHCIIa reached maximum accumulation at 28 DPA while LHCIIb reached maximum accumulation at 33 DPA. It is interesting to note that, unlike the other plant lines, all component Cab species in DIII reached maximum accumulation at 28 DPA. These results suggest that expression of LHCIIB may have been repressed in the DIII antisense cab transgenic line.

4.V.f: Correlations between total Cab and total chlorophyll (μg/seed)

In order to determine whether correlations existed between total chlorophyll and total Cab proteins for each of the 4 tested lines, correlation analysis was performed (Table 5). Twenty one pair-wise comparisons were used to calculate the correlation coefficient for each of the plant lines. All 4 lines, individually and collectively, showed a high probability of correlation between total Cab proteins and total chlorophyll during development. The high probability of correlation between Cab proteins and chlorophyll may indicate that the reduction in chlorophyll accumulation seen in the DIII line may be a result of reductions in Cab protein accumulation.

4.V.g: Effect of different variables on total Cab and total chlorophyll (μg/seed)

The effects of the variables used in this study were examined to determine which variable or combination of variables had a significant effect on chlorophyll and Cab protein accumulation in the seed. The variables examined included rep, DPA, plant line and two way interactions of these variables. Using a general linear model, it was observed that only the plant line and DPA had a significant effect on chlorophyll and Cab protein accumulation in the seed (Table 6). The effects of replicate, and the two way interactions of replicate by plant line, replicate by day and plant line by day did not appear to have a significant effect on chlorophyll or Cab protein accumulation in the seed.

4.VI: SEED MOISTURE

In order to determine whether or not the antisense cab gene had an effect on seed moisture content, the seed moisture content was determined throughout seed development in Westar, Control, DII and DIII plant lines. Maximum seed moisture was measured between 15 and 18 DPA (Fig. 22). Seed moisture slowly began to decrease after 18 DPA, reaching minimum measured seed moisture at 43 DPA. Seed moisture content was not examined after 43 DPA. Comparison of seed moisture for the 4 examined plant lines revealed no appreciable difference in seed moisture content.

4.VII: SEED DRY WEIGHT

The dry seed weights were examined throughout development in order to determine whether the pNABI construct had a deleterious effect (Fig. 23). Comparison of the plant lines at 15 DPA through 38 DPA showed no appreciable difference in the dry weights of the transgenic seeds compared to the Westar seed. However, at 43 the dry weight of DIII seed was considerably less compared to the Westar seed. The difference in mean dry weight/seed between Westar and DIII at 43 DPA is in excess of 1.3 mg/seed, which is equivalent to 28% of the dry weight of the Westar seed.

4.VIII: TOTAL SEED PROTEIN

4.VIII.a: Total protein (µg/seed)

Total protein (µg/seed) was also examined to determine whether or not the presence of the antisense cab construct had an effect on various aspects of seed development. A comparison of total protein (µg/seed) among Westar, Control, DII and DIII seed revealed little difference throughout seed development (Fig. 24). Maximum accumulation of total proteins was at 43 DPA for all four examined plant lines. When examined on a per seed basis, the pNABI construct did not appear to deleteriously affect total protein accumulation in transgenic seed.

4.VIII.b: Total protein (mg/gram dry seed weight)

Comparison of total protein (mg/gram dry weight) revealed differences not seen in the comparison of total protein/seed among the four plant lines (Fig. 25). At each of the examined DPA, protein levels for DII appeared to be higher relative to Westar. DIII protein levels were also higher than in Westar at 15, 18, 28, 33, and 43 DPA.

Plant Line	Number of Plants Tested	Plants with Gus activity	Plants without Gus activity
Westar	10	0	10
Control	10	0	10
DII	24	21	3
DIII	24	24	0

TABLE 1: Histochemical Gus assay of seedlings of Westar and Control, and T2 seedlings of DII and DIII. The seeding leaf tissue was vacuum infiltrated in X-gluc solution for 30 minutes, then incubated overnight at 37 degrees Celsius. After bleach treatment to remove pigment, the samples were scored for presence or absence of blue tissue. Those samples displaying blue tissue were scored as having positive Gus activity. Samples showing no evidence of blue tissue were scored as having no Gus activity. The ratio of Gus positive: Gus negative samples within one line suggested the level of homozygosity of pNABI attained in the transgenics.

Plant Line	Time of Maximum Chlorophyll Accumulation	Time of Maximum Cab Protein Accumulation
Westar	33 DPA	28 DPA
Control	28 DPA	28 DPA
DII	28 DPA	28-33 DPA
DIII	28 DPA	28 DPA

TABLE 2: Comparison of chlorophyll and Cab protein accumulation during seed development. Western blots were probed with α-CPIa antibody were analyzed using densitometry in order to determine the point during seed development that Cab proteins reached maximum accumulation. Chlorophyll was extracted in 80% acetone from seed samples harvested at various stages of maturity. Extracted chlorophyll was quantified in order to determine the point at which maximum chlorophyll accumulation occurred during seed development.

TABLE 3: Comparison of chlorophyll and Cab accumulation in seed of Westar and Control, and T2 seed of DII and DIII. **A:** Chlorophyll accumulation in seed of Westar and Control, and T2 seed of DII and DIII. Chlorophyll was extracted in 80% acetone from seed samples harvested at various stages of maturity. Extracted chlorophyll was quantified (μg/seed) and the least significant difference for each DPA was calculated at a 0.1 confidence level. Means with the same letter are not significantly different. **B:** Cab accumulation in seed of Westar and Control, and T2 seed of DII and DIII. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa detected with 2 ° antibody conjugated to alkaline phosphatase. Resulting western blots were analyzed using densitometry in order to quantify Cab accumulation (Volume [OD/mm^2] x 10) in seed harvested at various stages of maturity. Least significant difference was calculated for each DPA at a 0.1 confidence level. Means with the same letter are not significantly different.

A:

	Westar	Control	DII	DIII
15 DPA	0.4 (a)	0.3 (a)	0.5 (a)	0.3 (a)
18 DPA	1.0 (b)	0.9 (b)	1.3 (b)	0.7 (b)
23 DPA	2.5 (c)	2.2 (c)	2.4 (c)	1.9 (c)
28 DPA	3.6 (d)	3.1 (d)	3.2 (d)	3.0 (d)
33 DPA	3.9 (e)	2.7 (f)	2.5 (f)	2.0 (f)
38 DPA	2.9 (g)	2.5 (g/h)	1.7 (h)	1.8 (h)
43 DPA	1.4 (i/j)	1.6 (i)	0.9 (i/j)	0.7 (j)

B:

	Westar	Control	DII	DIII
15 DPA	19 (z)	21 (z)	13 (z)	22 (z)
18 DPA	7 (y)	13 (y)	8 (y)	2 (y)
23 DPA	88 (x/w)	106 (x)	106 (x)	45 (w)
28 DPA	141 (v/u)	157 (v)	137 (u)	112 (t)
33 DPA	116 (s/r)	123 (s)	117 (s/r)	100 (r)
38 DPA	64 (q)	55 (q)	48 (q)	49 (q)
43 DPA	25 (o)	57 (p)	32 (o)	15 (o)

TABLE 4: Comparison of significant differences in Cab accumulation between seed of Westar, Control, and T2 seed of DIII. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Resulting western blots were analyzed using densitometry in order to quantify Cab accumulation (Volume {OD/mm^2 } x 10) in seed harvested at various stages of maturity. The least significant difference was calculated for each DPA at a 0.1 confidence level. Percentage of the least significant difference was calculated from the difference between the means of Westar and DIII or Control and DIII at each examined DPA divided by the least significant difference. The percentage of the least significant difference was calculated to indicate the extent to which Westar and Control accumulated higher Cab levels than DIII. For instance, at 38 DPA, 78% indicates that Cab accumulation in Westar exceeded Cab accumulation in DIII by 78% of the least significant difference. Least significant difference for each DPA is also listed.

	Westar/DIII	Control/DIII	Least Significant Difference
15 DPA	0%	0%	27.4
18 DPA	20%	25%	25.2
23 DPA	91%	100%	47.7
28 DPA	100%	100%	18.9
33 DPA	84%	100%	19.4
38 DPA	78%	31%	19.2
43 DPA	53%	100%	18.8

	Pearson correlation coefficient	Pr
Wester	0.868	0.0005
Control	0.833	0.0001
DII	0.876	0.0001
DIII	0.848	0.0001

TABLE 5: Correlations between total Cab and total chlorophyll (µg/seed). The Pearson correlation coefficient was calculated based on 21 pair-wise comparisons for each of the four plant lines to determine the correlation between Cab and chlorophyll accumulation during seed development. A correlation coefficient of 1 indicates the highest degree of correlation possible. Pr indicates the probability level of the Pearson correlation coefficient.

TABLE 6: Comparison of the effects of variables and two way variable interactions on total chlorophyll and total Cab protein accumulation in seed. **A:** A general linear model was used to calculate the probability of each examined variable or two-way variable interaction having a significant effect on total chlorophyll accumulation in seed. Pr>F indicates the probability of the examined variable having a significant effect on chlorophyll accumulation. **B:** A general linear model was used to calculate the probability of each examined variable or two-way variable interaction having a significant effect on total Cab protein accumulation in seed. Pr>F indicates the probability of the examined variable having a significant effect on Cab protein accumulation. As Pr>F approaches 0, the probability of a significant effect increases.

A:

Variable	**F Value**	**Pr>F**
Rep	0.58	0.5671
Plant Line	7.63	0.0004
DPA	48.36	0.0001
Rep*Line	0.99	0.449
Rep*DPA	1.15	0.3565
Line*DPA	1.3	0.2474

B:

Variable	**F Value**	**Pr>F**
Rep	0.08	0.7769
Plant Line	5.52	0.0072
DPA	78.12	0.0001
Rep*Line	0.49	0.6919
Rep*DPA	1.28	0.3134
Line*DPA	2.35	0.0391

FIG. 3: Histochemical Gus assay performed on leaf tissue from putative transgenics. Development of blue pigment in tissues indicated positive Gus activity. (1) CII; (2) DI; (3) DII; (4) DIII; (5) positive control (*Brassica napus* containing Gus gene); (6) *Brassica napus* cv. Westar.

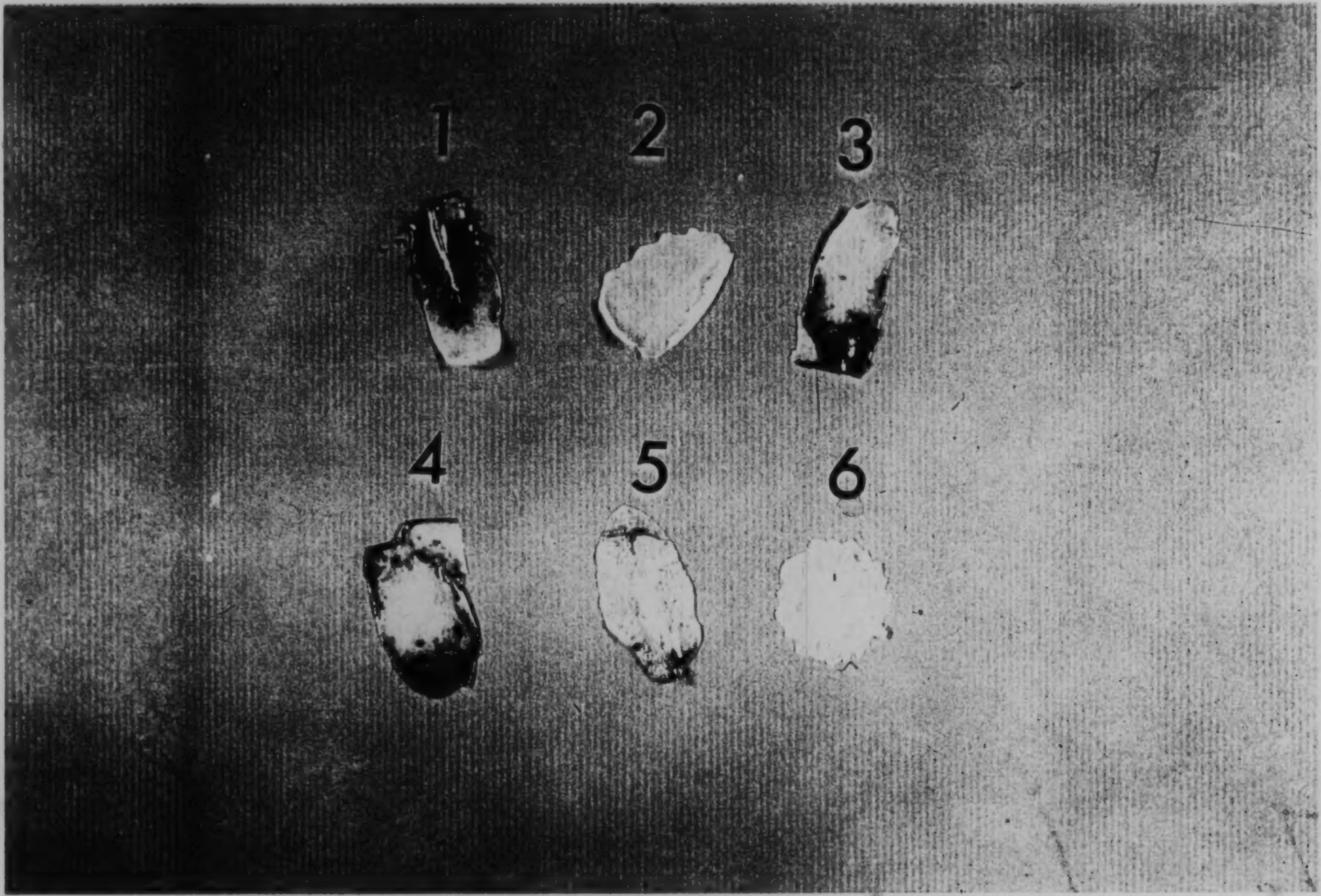

1
2
3
4
5
6

FIG. 4: Agarose gel showing results of polymerase chain reaction (PCR) amplification of the nptII fragment of the pNABI construct. 1 µg of DNA extracted from leaf tissue of putative transgenics, Westar and Control was used as the PCR template. The presence of a 0.7 Kb fragment indicated successful amplification. The presence on an extra low molecular weight band in lane 4 in likely the result of dimer formation between excess primers. (1) 1 Kb ladder; (2) CII ; (3) non-transformant; (4) DII; (5) DIII; (6) positive control (*Brassica napus* transformed with FV4 vector [contains the Gus gene]); (7) Westar; (8) 1 Kb ladder.

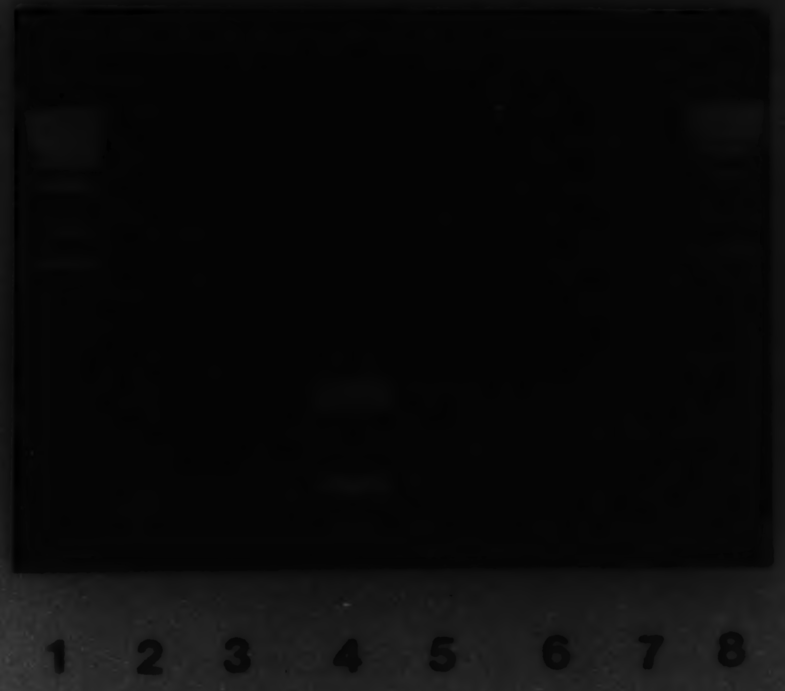
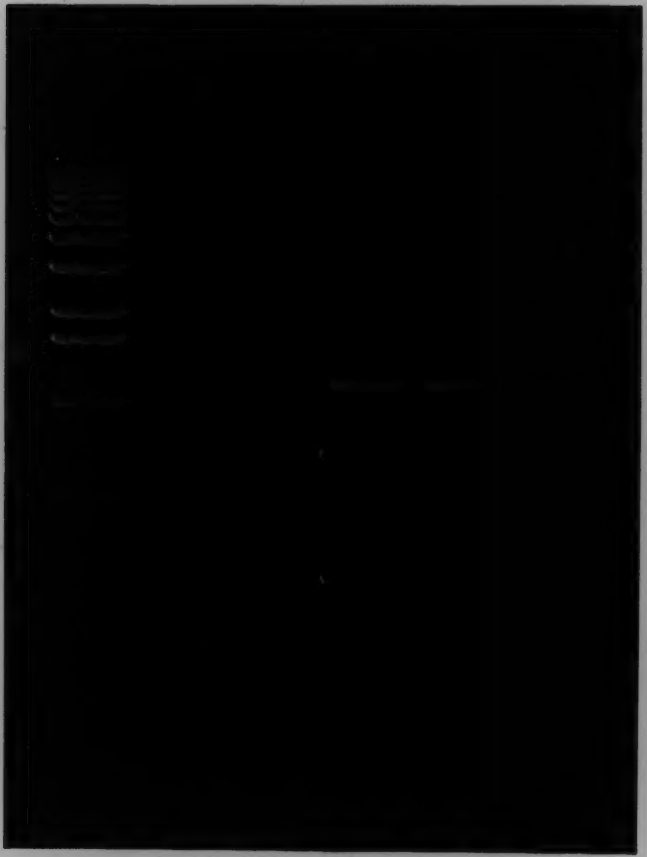

1
2
3
4
5
6
7
8

FIG. 5: Agarose gel showing results of PCR amplification using Gus primers. 1 µg of DNA extracted from leaf tissue was used as the PCR template. The presence of 1.2 Kb fragment indicated successful amplification of the Gus gene in the pNABI construct. (1) 1 Kb ladder; (2) Westar; (3) Control; (4) DII; (5) DIII; (6) BINSYN plasmid containing pNABI construct.



1 2 3 4 5 6

FIG. 6: Southern blot analysis identifying the number of copies of the pNABI construct in seed of transgenic *Brassica napus*. 15 µg of T2 leaf DNA were cleaved with Pst I and hybridized with a radiolabelled 2.75 Kb Bam HI fragment of the Gus/nptII coding region. Detection of two bands (1.7 and 2.1 Kb) indicated the presence of a single copy of the pNABI construct in DIII and DII lines. (1) DIII; (2) DII; (3) Control; (4) Westar.

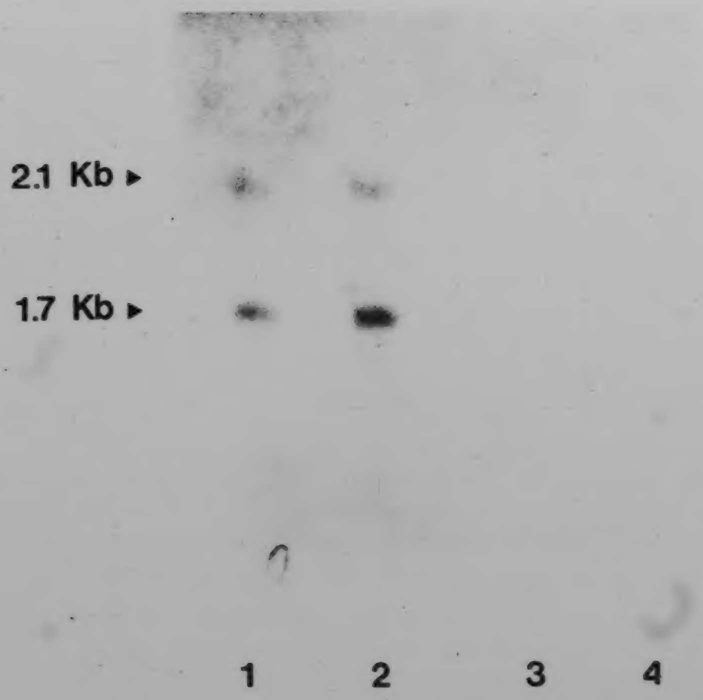

2.1 Kb ▸
1.7 Kb ▸
1
2
3
4

FIG. 7: Agarose gel showing results of Pst I restriction of the 2.75 Kb Bam HI Gus/nptII probe used for Southern blot analysis. 2 µg of purified Bam HI fragment were cleaved by Pst I. The presence of 1.1 and 1.6 Kb fragments revealed that the Bam HI 2.75 Kb fragment is cleavable by Pst I. (1) 1 Kb ladder; (2) 2 µg of 2.75 Kb Bam HI Gus/nptII fragment from pNABI restricted with Pst I; (3) 2 µg of uncut 2.75 Kb Bam HI Gus/nptII fragment from pNABI construct.

1
2
3

FIG. 8: The effect of antisense cab on total chlorophyll in transgenic *Brassica napus*. Seed from transgenics, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone. Values are the mean ± S.E., n=3.

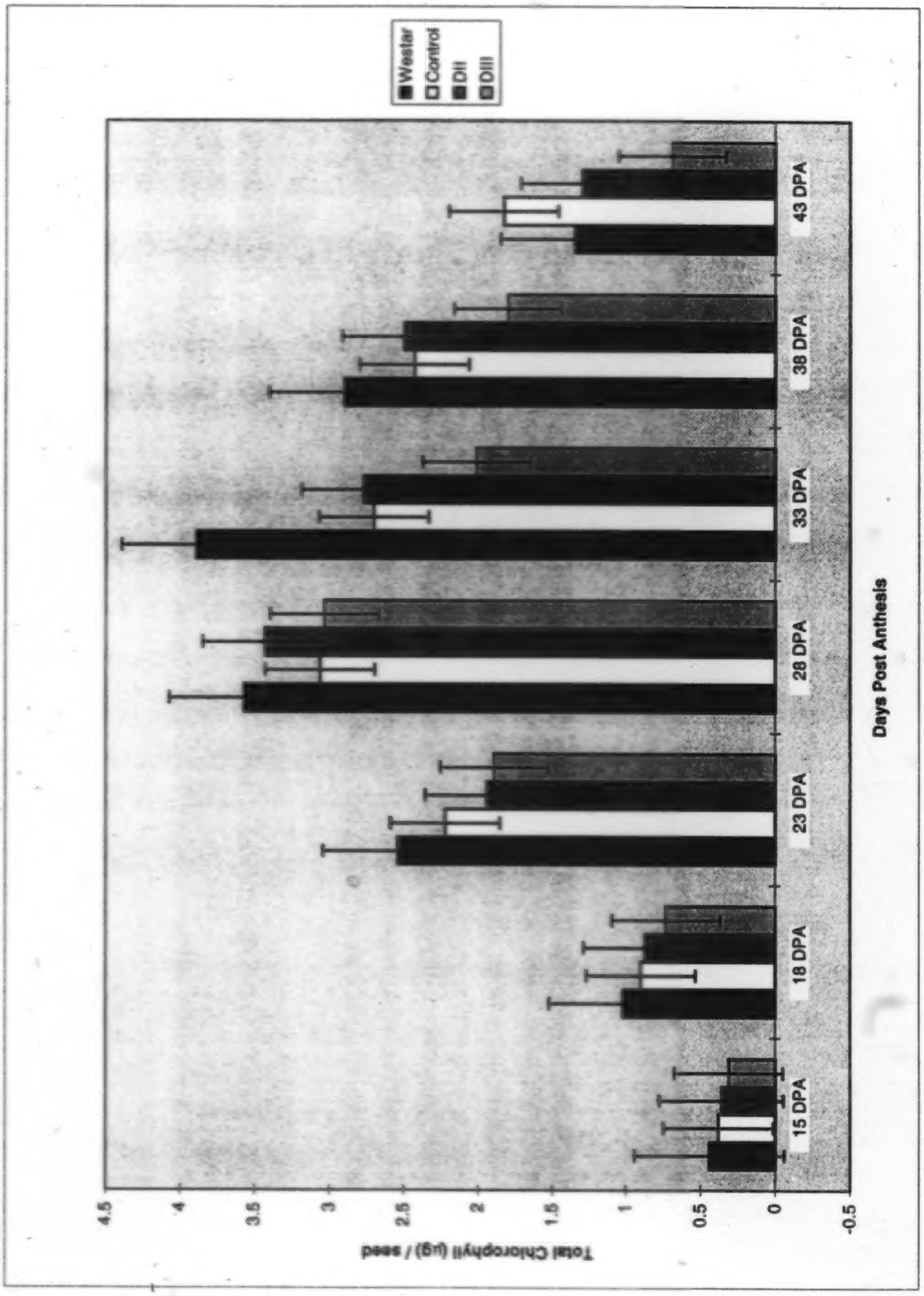

Total Chlorophyll (µg) / seed
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
Westar
Control
DII
DIII

FIG. 9: The effect of antisense cab on chlorophyll a per seed of transgenic *Brassica napus*. Seed from DII, DIII, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone. Values are the mean ± the standard error (S.E.), n=3.

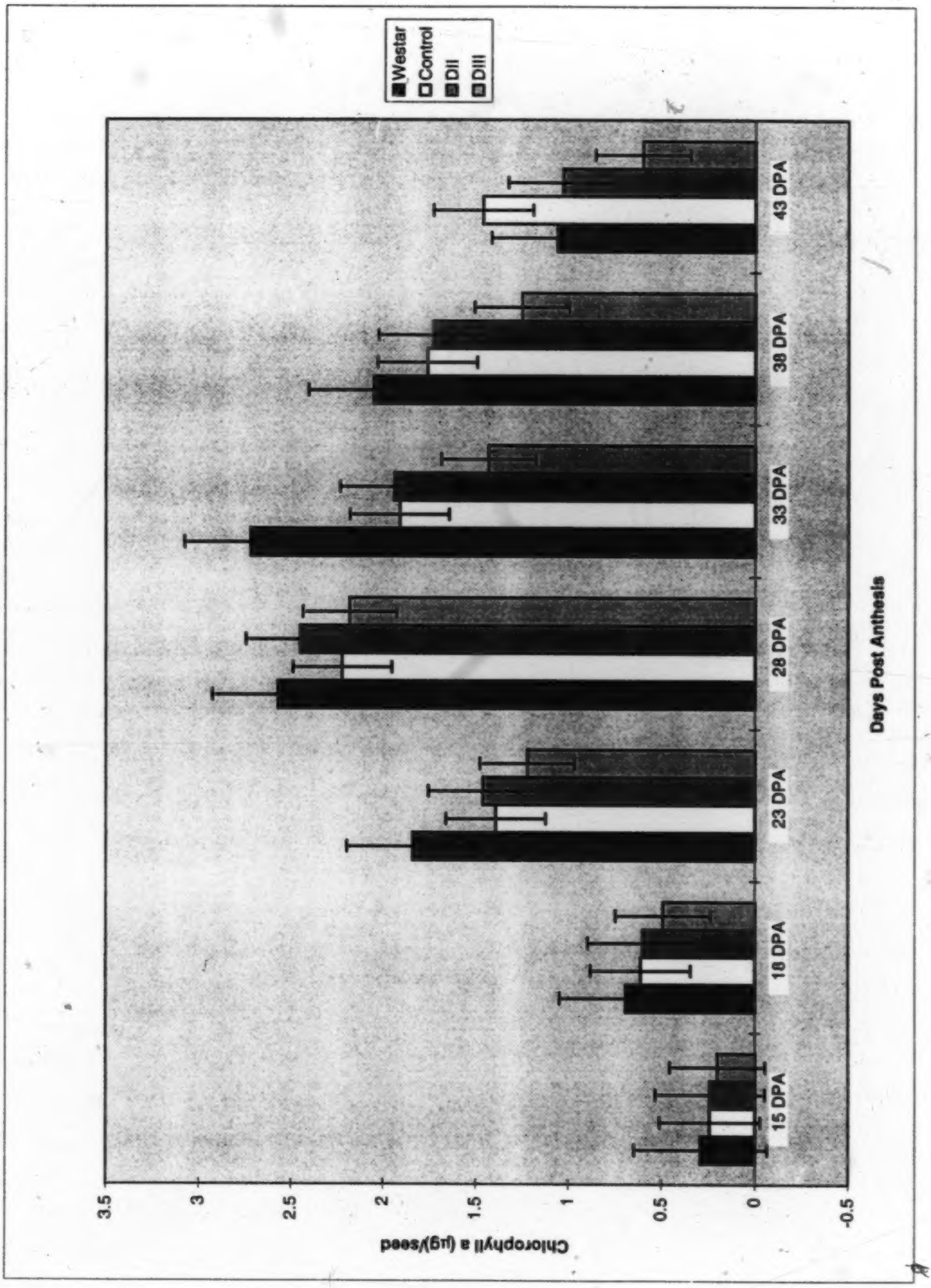

Chlorophyll a (µg)/seed
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
Westar
Control
DII
DIII

FIG. 10: The effect of antisense cab on chlorophyll b in transgenic *Brassica napus*. Seed from transgenic, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone. Values are the mean ± S.E., n=3.

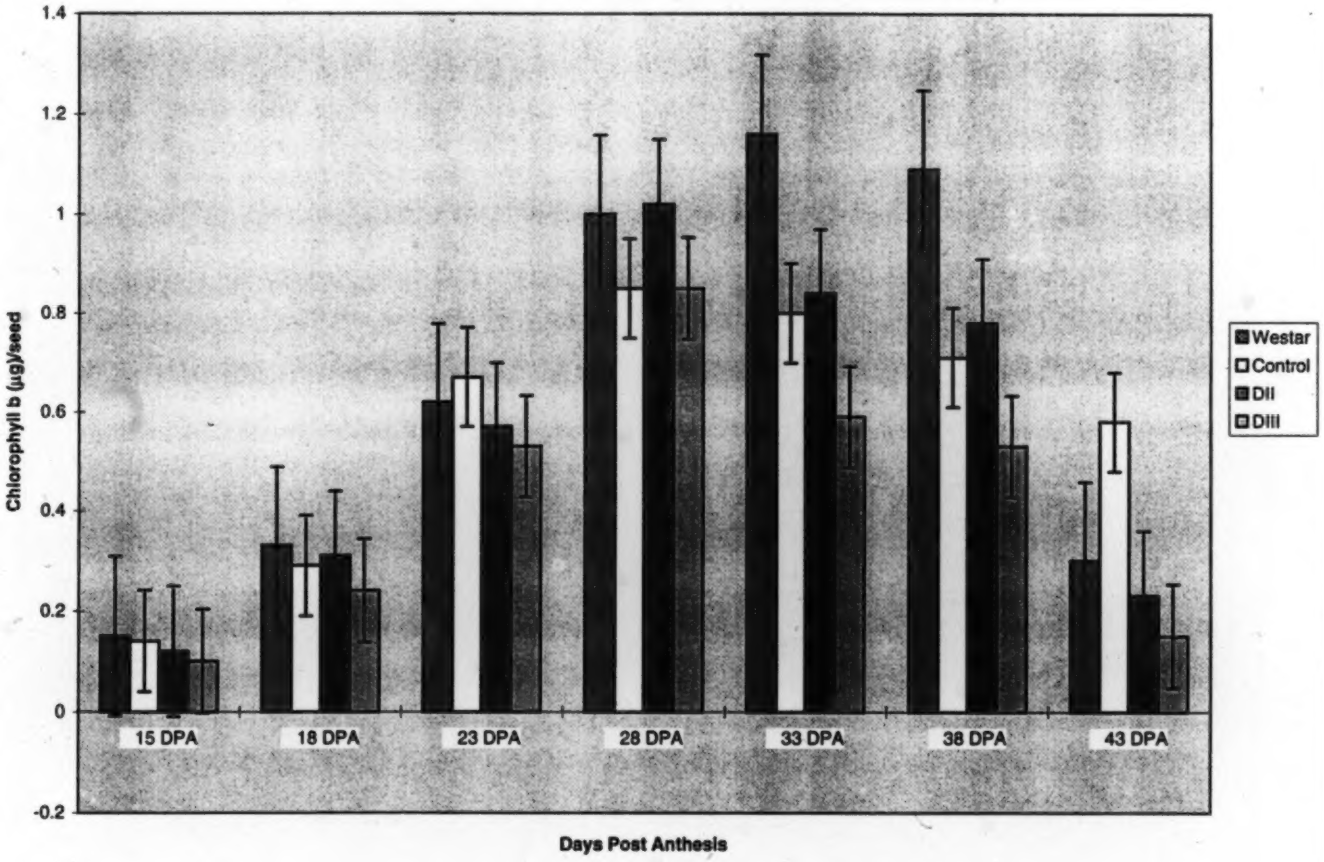

1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Chlorophyll b (µg)/seed
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
▨ Westar
☐ Control
▨ DII
▨ DIII

FIG. 11: The effect of antisense cab on chlorophyll a per gram dry seed weight in developing seed of transgenic *Brassica napus*. Seed from transgenics, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone. Values are the mean ± S.E., n=3.

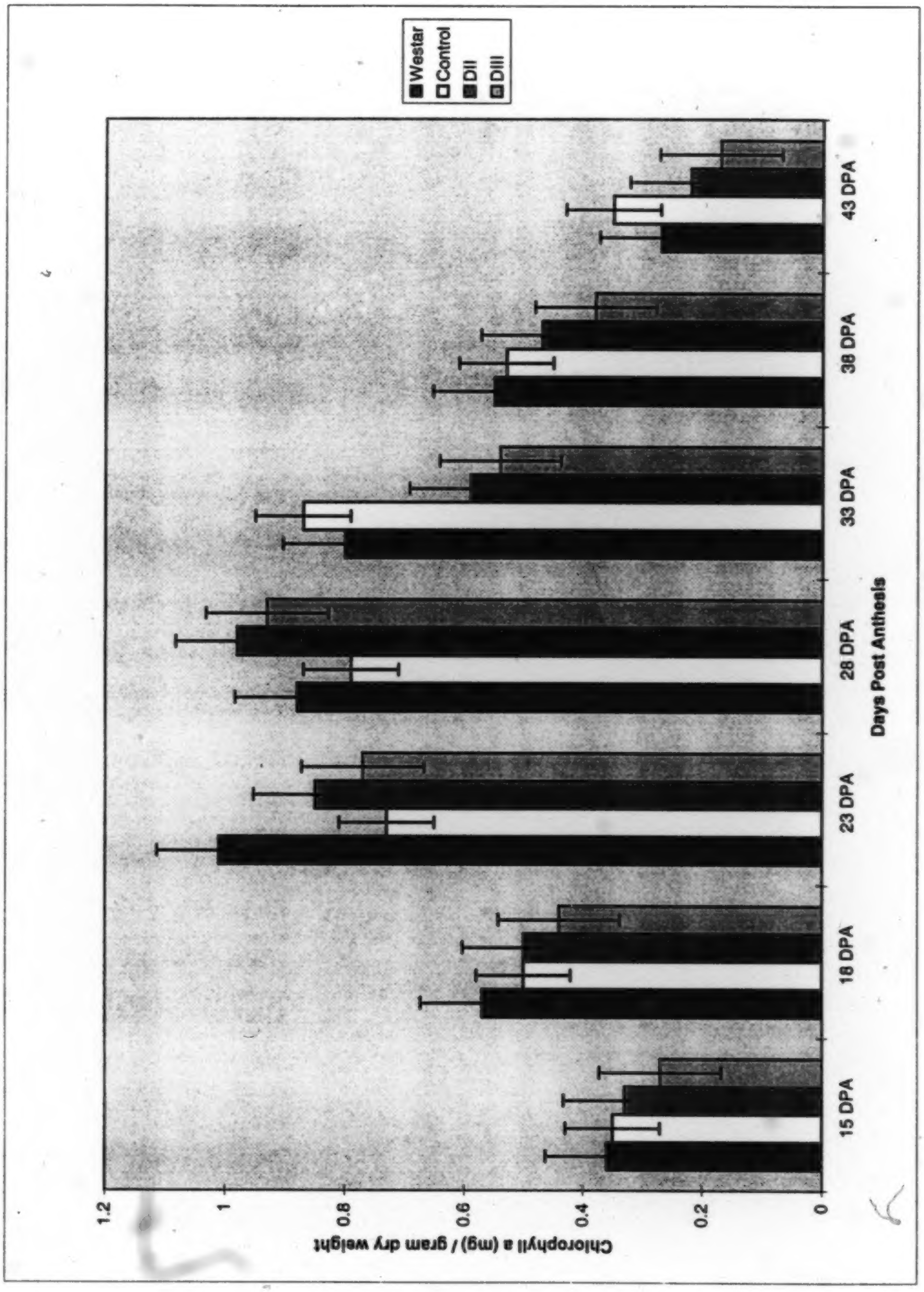

Chlorophyll a (mg) / gram dry weight
0
0.2
0.4
0.6
0.8
1
1.2
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
■ Westar
☐ Control
▨ DII
▨ DIII

FIG. 12: The effect of antisense cab on chlorophyll b per gram dry seed weight in transgenic *Brassica napus*. Seed from transgenics, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone. Values are the mean ± S.E., n=3.

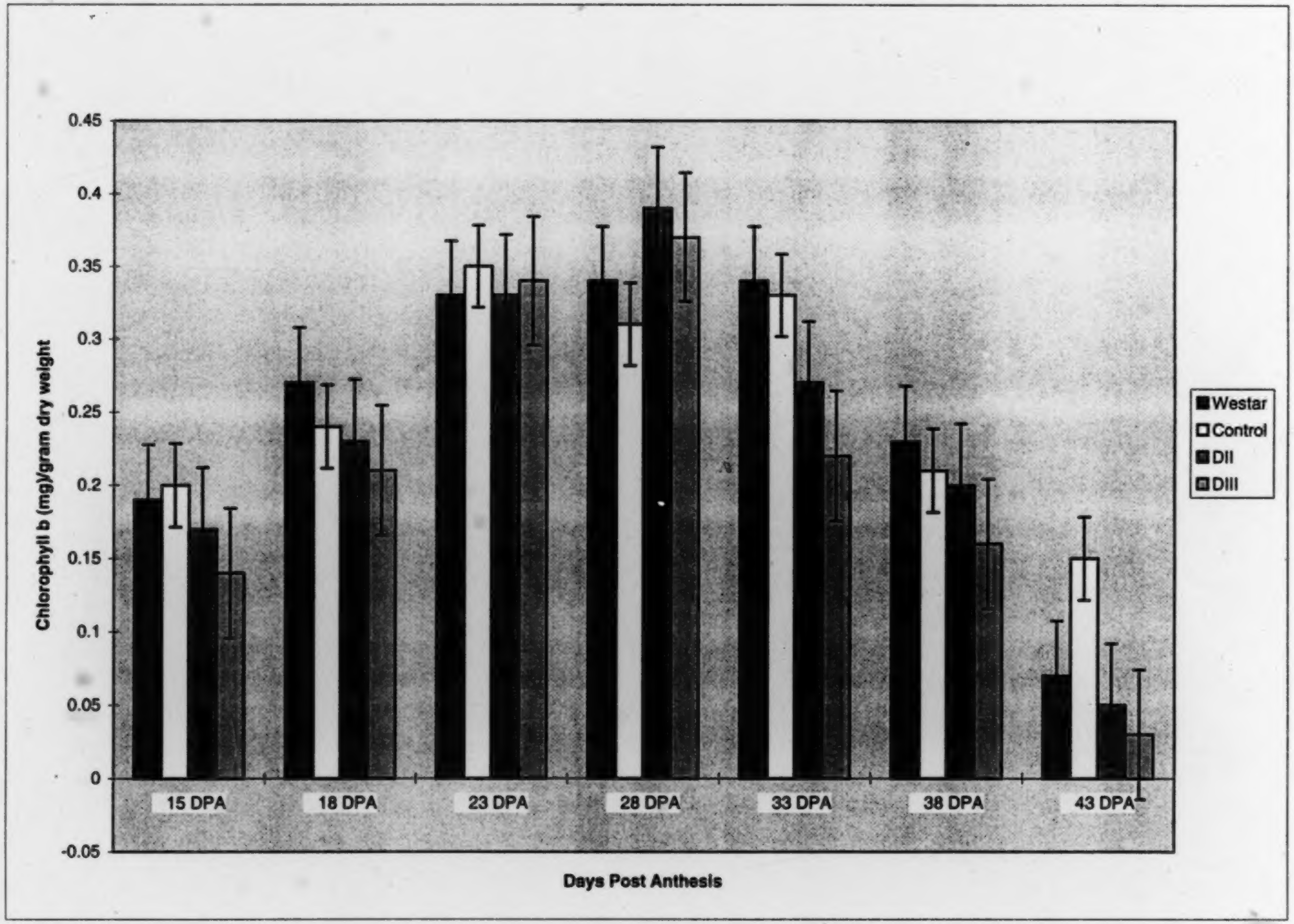

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
Chlorophyll b (mg)/gram dry weight
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
■ Westar
☐ Control
■ DII
▨ DIII

FIG. 13: The effect of antisense cab on total chlorophyll per gram dry seed weight in transgenic *Brassica napus*. Seed from transgenics, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone. Values are the mean ± S.E., n=3.

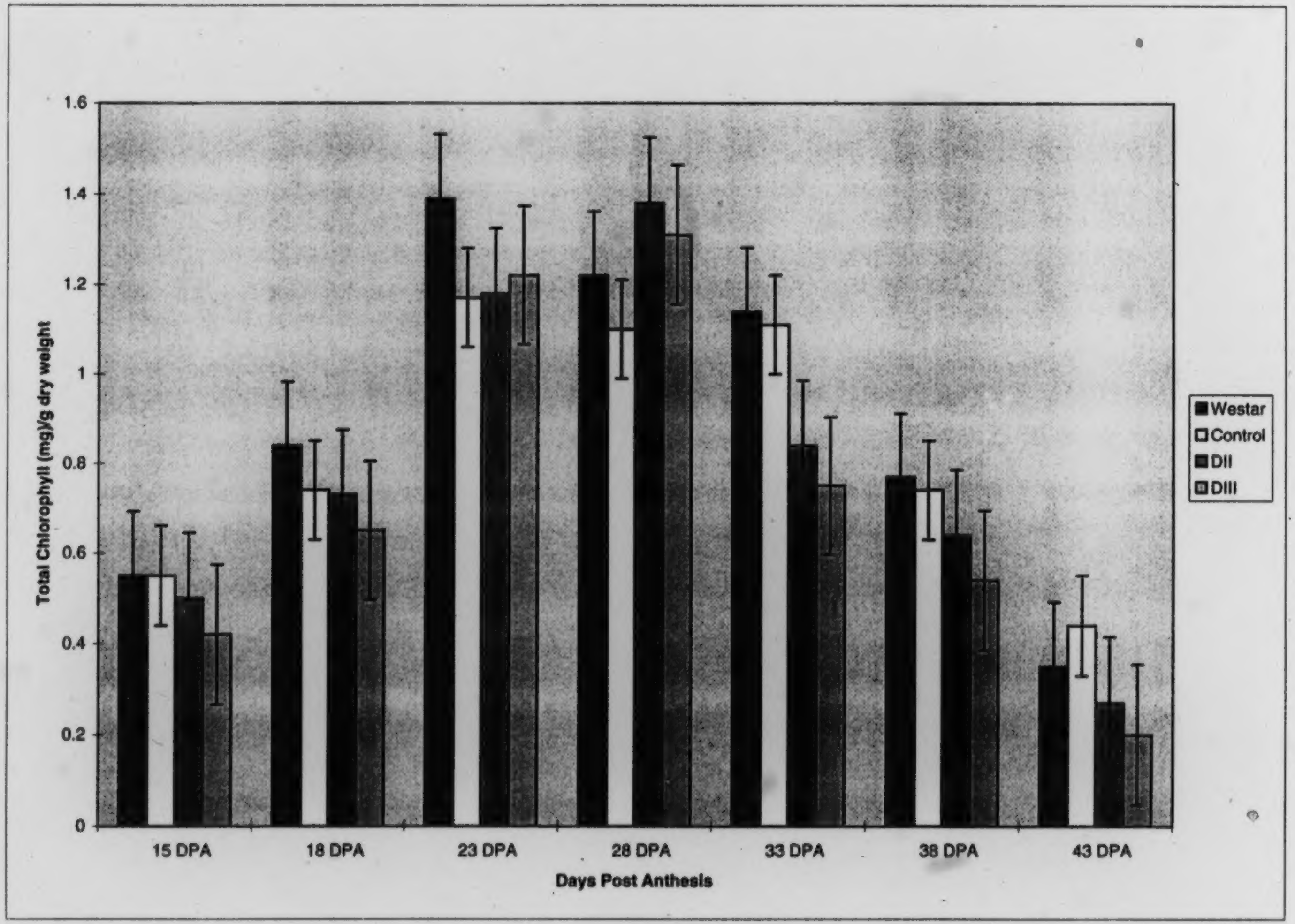

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Total Chlorophyll (mg)/g dry weight
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
Westar
Control
DII
DIII

FIG. 14: The effect of antisense cab on total chlorophyll per gram total protein in transgenic *Brassica napus*. Seed from transgenics, Westar and Control *Brassica napus* were harvested at 7 different DPA. Chlorophyll was extracted in 80% acetone from seed harvested at 7 different DPA. Chlorophyll extractions were repeated three times. Total seed protein was calculated based on known protein concentrations, volumes, dry seed weight and numbers of seed used for each extraction. Values represent the means ± S.E., n=3.

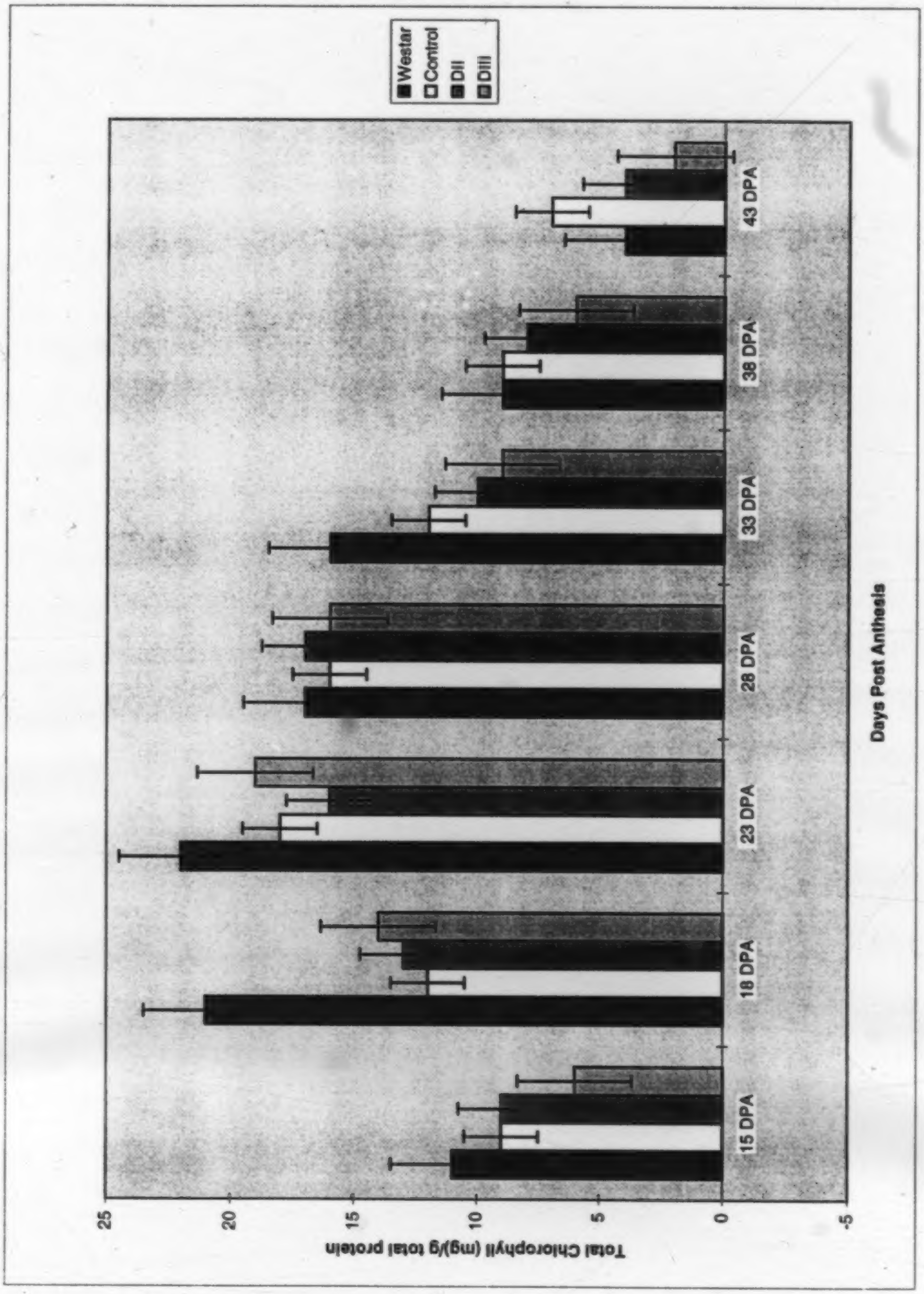

Total Chlorophyll (mg)/g total protein
25
20
15
10
5
0
-5
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
Westar
Control
DII
DIII

FIG. 15: The effect of antisense cab of the chlorophyll a/b ratios in seed of transgenic *Brassica napus*. Chlorophyll was extracted in 80% acetone from seed harvested at 7 different DPA. Values represent the mean ± S.E., n=3.

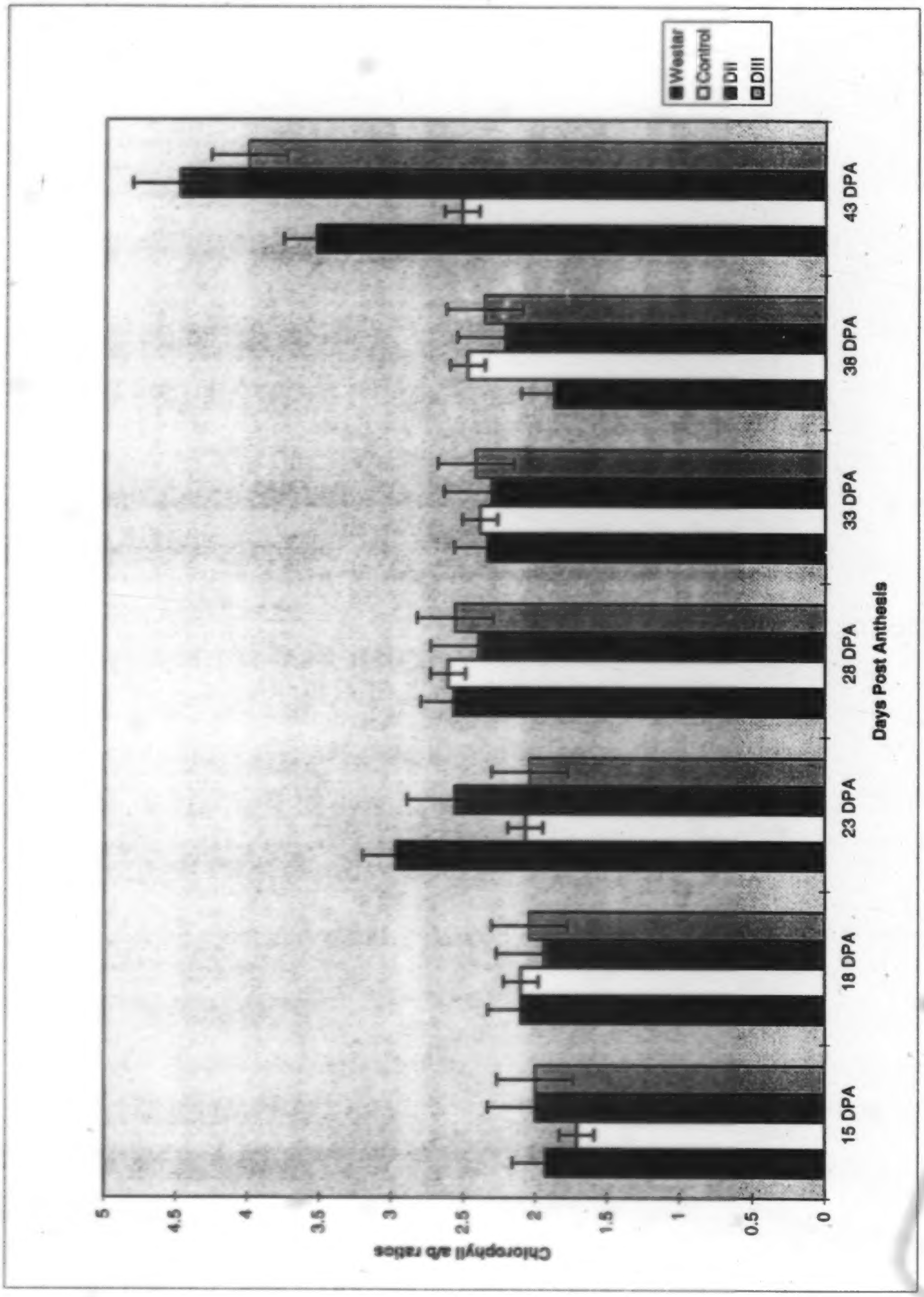

Chlorophyll a/b ratios
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
▨ Westar
☐ Control
■ DII
▨ DIII

FIG. 16: Western blot analysis of the accumulation of Cab polypeptides during seed development in *Brassica napus* cv. Westar. Total acetone precipitable proteins were extracted from a known number of seed at 7 different DPA. Total protein was separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. LHCIIa is visible at approximately 29 Kd. The LHCIIb polypeptide is visible at approximately 26 Kd. The four bands making up LHCI are visible between 20 and 24 Kd. Samples were loaded on a per seed basis (0.5 seed/lane). Lanes are as follows: (1) 15 DPA; (2) 18 DPA; (3) 23 DPA; (4) 28 DPA; (5) 33 DPA; (6) 38 DPA; (7) 43 DPA; (8) Molecular weight standard. 30 K refers to the apparent molecular weight of 30 Kd.

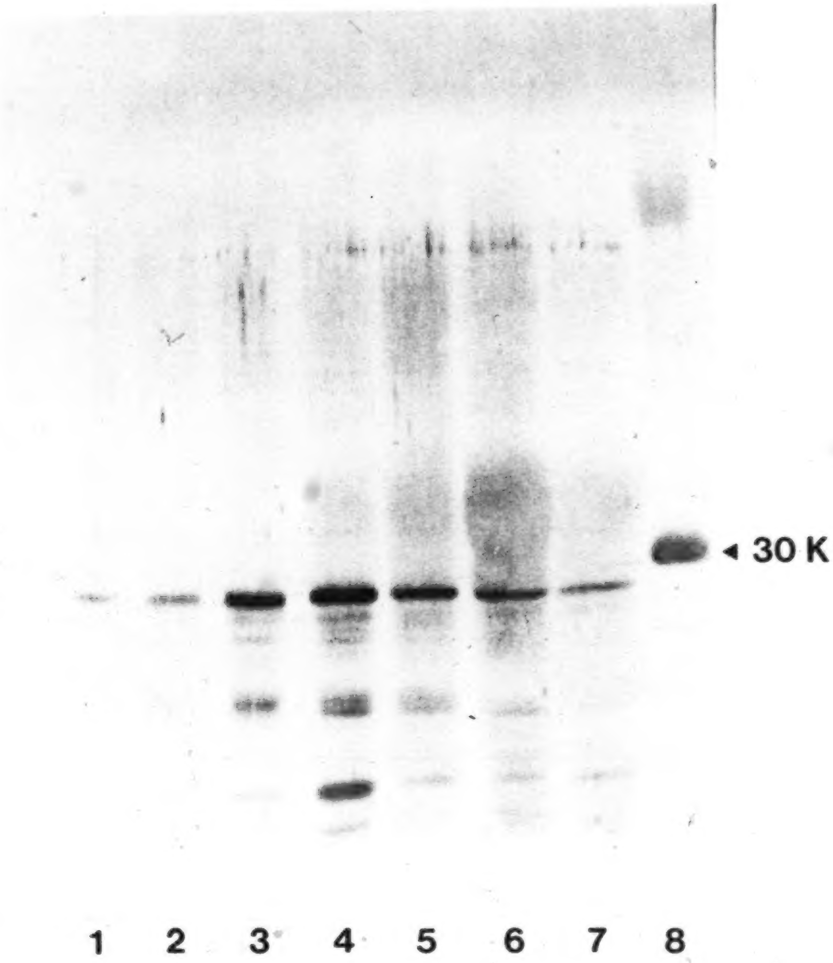

30 K
1
2
3
4
5
6
7
8

FIG. 17: Western blot analysis of the accumulation of Cab polypeptides during seed development in Control plants derived from *Brassica napus* cv. Westar. Total acetone precipitable proteins were extracted from a known number of seed at 7 different DPA. Total protein was separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. LHCIIa is visible at approximately 29 Kd. The LHCIIb polypeptide is visible at approximately 26 Kd. The four bands making up LHCI are visible between 20 and 24 Kd. Samples were loaded on a per seed basis (0.5 seed/lane). Lanes are as follows: (1) 15 DPA; (2) 18 DPA; (3) 23 DPA; (4) 28 DPA; (5) 33 DPA; (6) 38 DPA; (7) 43 DPA; (8) Molecular weight standard. 30 K refers to the apparent molecular weight of 30 Kd.

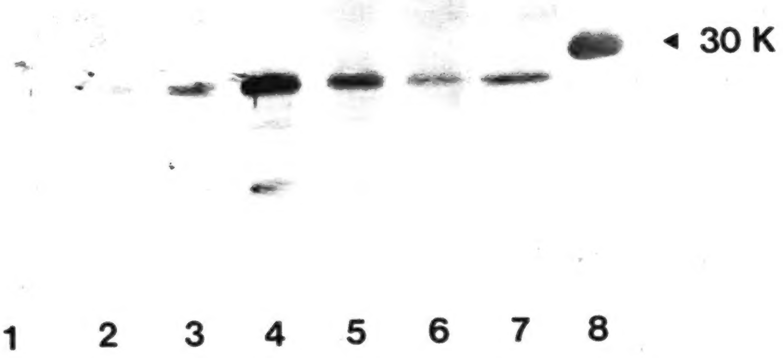

◂ 30 K
1
2
3
4
5
6
7
8

FIG. 18: Western blot analysis of the accumulation of Cab polypeptides during seed development in the antisense cab transgenic, DII. Total acetone precipitable proteins were extracted from a known number of T2 seed at 7 different DPA. Total protein was separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. LHCIIa is visible at approximately 29 Kd. The LHCIIb polypeptide is visible at approximately 26 Kd. The four bands making up LHCI are visible between 20 and 24 Kd. Samples were loaded on a per seed basis (0.5 seed/lane). Lanes are as follows: (1) 15 DPA; (2) 18 DPA; (3) 23 DPA; (4) 28 DPA; (5) 33 DPA; (6) 38 DPA; (7) 43 DPA; (8) Molecular weight standard. 30 K and 14 K refer to apparent molecular weights of 30 Kd and 14 Kd, respectively.

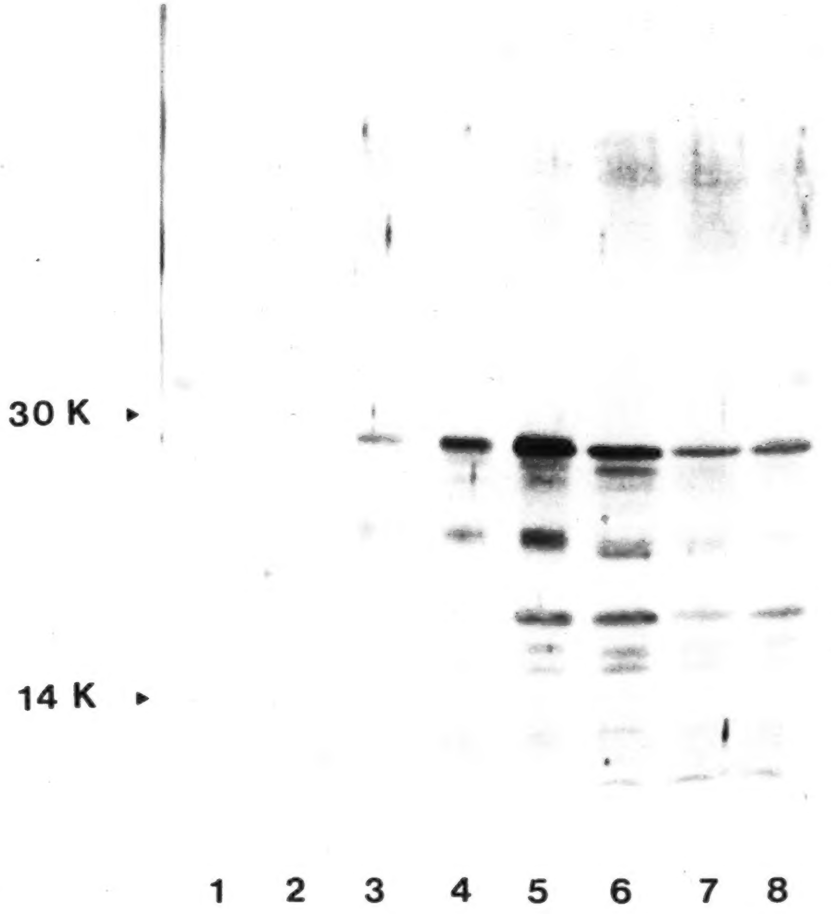

30 K
14 K
1
2
3
4
5
6
7
8

FIG. 19: Western blot analysis of the accumulation of Cab polypeptides during seed development in the antisense cab transgenic, DIII. Total acetone precipitable proteins were extracted from a known number of T2 seed at 7 different DPA. Total protein was separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose, probed with α-CPIa and antigenic polypeptides detected with 2° antibody conjugated to alkaline phosphatase. LHCIIa is visible at approximately 29 Kd. The LHCIIb polypeptide is visible at approximately 26 Kd. The four bands making up LHCI are visible between 20 and 24 Kd. Samples were loaded on a per seed basis (0.5 seed/lane). Lanes are as follows: (1) 15 DPA; (2) 18 DPA; (3) 23 DPA; (4) 28 DPA; (5) 33 DPA; (6) 38 DPA; (7) 43 DPA; (8) Molecular weight standard. 30 K and 14 K refer to apparent molecular weights of 30 Kd and 40 Kd, respectively.

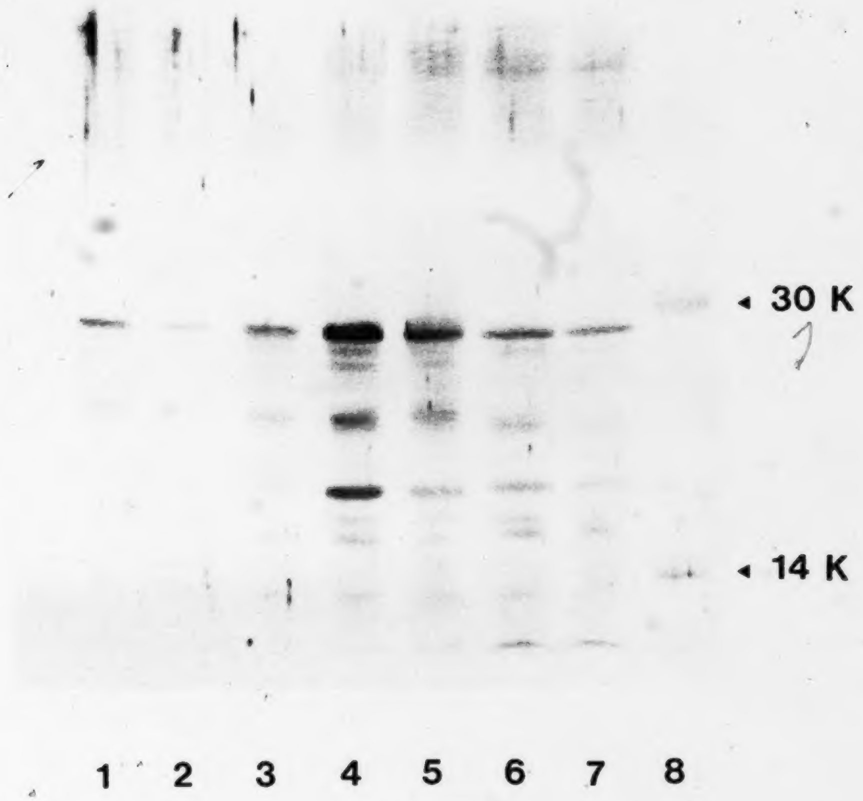

30 K
14 K
1
2
3
4
5
6
7
8

FIG. 20: Western blot analysis of the accumulation of Cab polypeptides in seed of Westar and Control, and T2 seed of DII and DIII at 23 DPA. Samples were loaded on an equal total protein basis (100 µg/lane). Total protein was separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. LHCIIa is visible at approximately 29 Kd. The LHCIIb polypeptide is visible at approximately 26 Kd. The four bands making up LHCI are visible between 20-24 Kd. Lanes are as follows: (1) Westar; (2) Control; (3) DII; (4) DIII; (5) Molecular weight standard. 30 K and 14 K refer to apparent molecular weights of 30 Kd and 14 Kd, respectively.

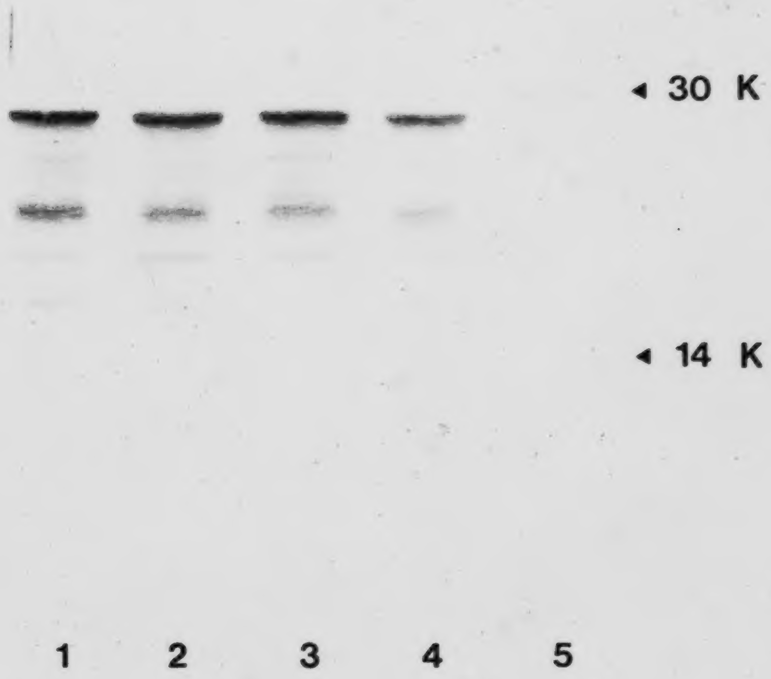

◂ 30 K
◂ 14 K
1
2
3
4
5

FIG. 21: Western blot analysis of the accumulation of Cab polypeptides in seed of Westar and Control, and T2 seed of DII and DIII at 18 DPA. Samples were loaded on a per seed basis (0.5 seed/lane). Total acetone precipitable proteins were extracted from a known number of T2 seed. Total protein was separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. LHCIIa is visible at approximately 29 Kd. The LHCIIb polypeptide is visible at approximately 26 Kd. The four bands making up LHCI are visible between 20-24 Kd. Lanes are as follows: (1) Westar; (2) Control; (3) DII; (4) DIII; (5) Molecular weight standard. 30 K and 14 K refer to apparent molecular weights of 30 Kd and 14 Kd, respectively.

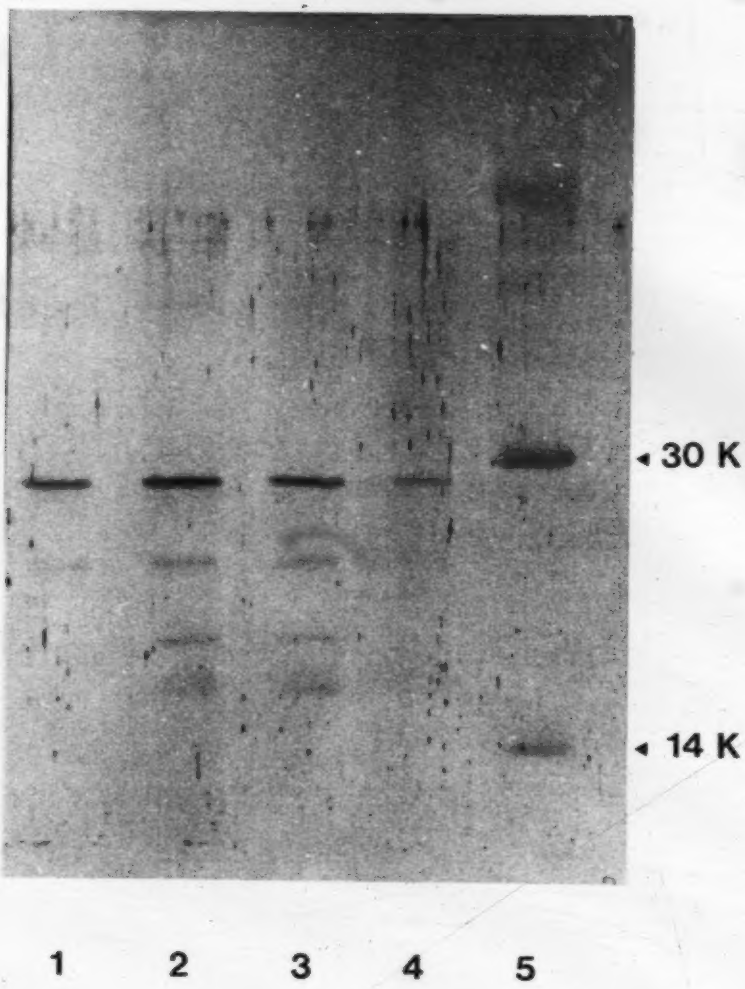

30 K
14 K
1
2
3
4
5

FIG. 22: Effect of antisense cab on seed moisture content during seed development in transgenic *Brassica napus*. Seed samples from Westar, Control, DII and DIII plants were harvested at 7 different DPA. The fresh weight of samples containing 20 or 30 seed were measured. The seed samples were then dried and weighed to obtain the dry seed weight. The difference between the dry and fresh weight calculated on per seed basis provided the seed moisture content. Values represent the mean percent seed moisture ± S.E., n=3.

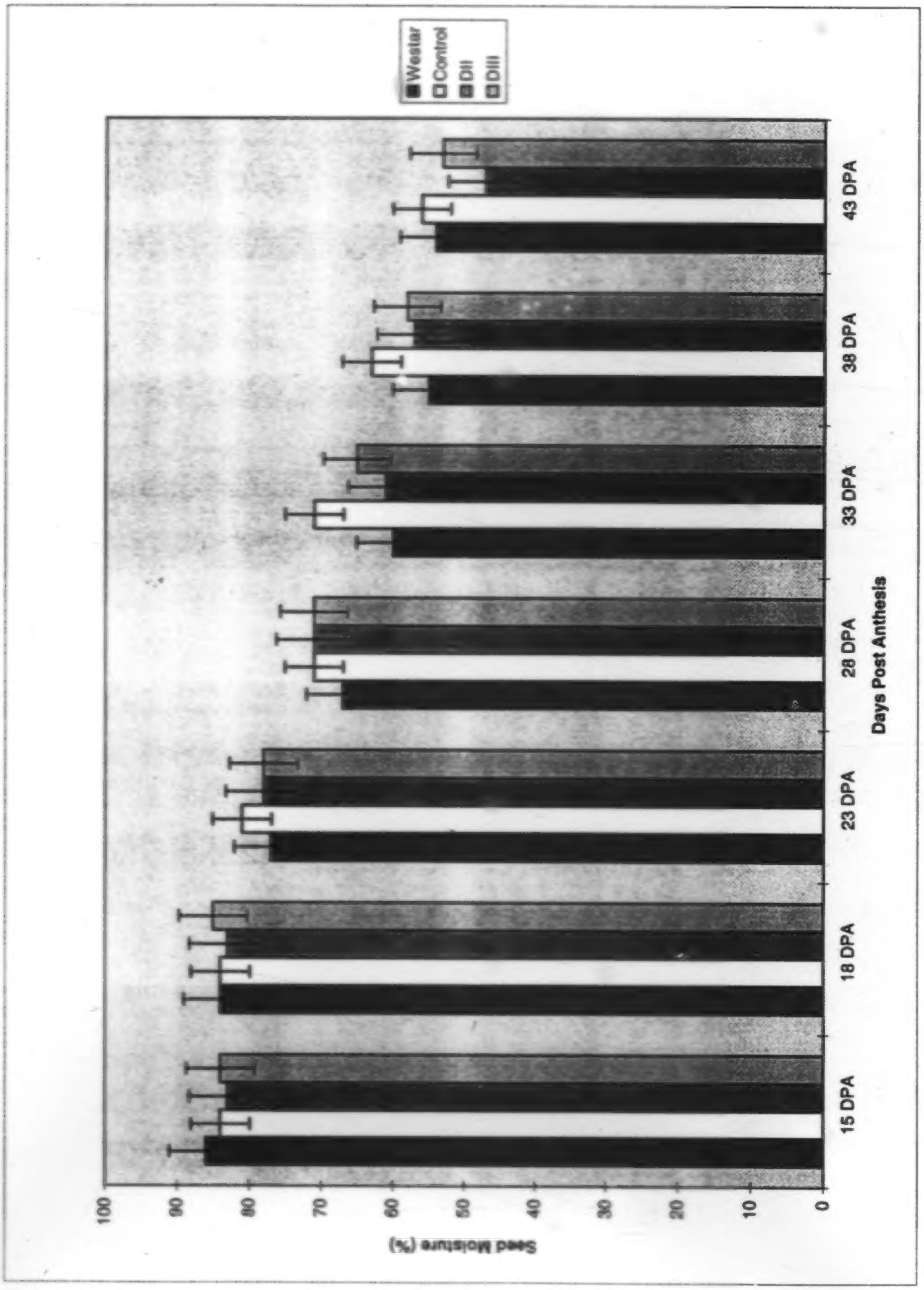

Seed Moisture (%)
100
90
80
70
60
50
40
30
20
10
0
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
■ Westar
☐ Control
▨ DII
▨ DIII

FIG. 23: Effect of antisense cab on dry seed weight during seed development in transgenic *Brassica napus*. Seed samples from Westar, Control, DII and DIII plants were harvested at 7 different DPA. Samples of 20 or 30 seed were dried and weighed to obtain the dry seed weight. Values represent the mean ± S.E., n=3.

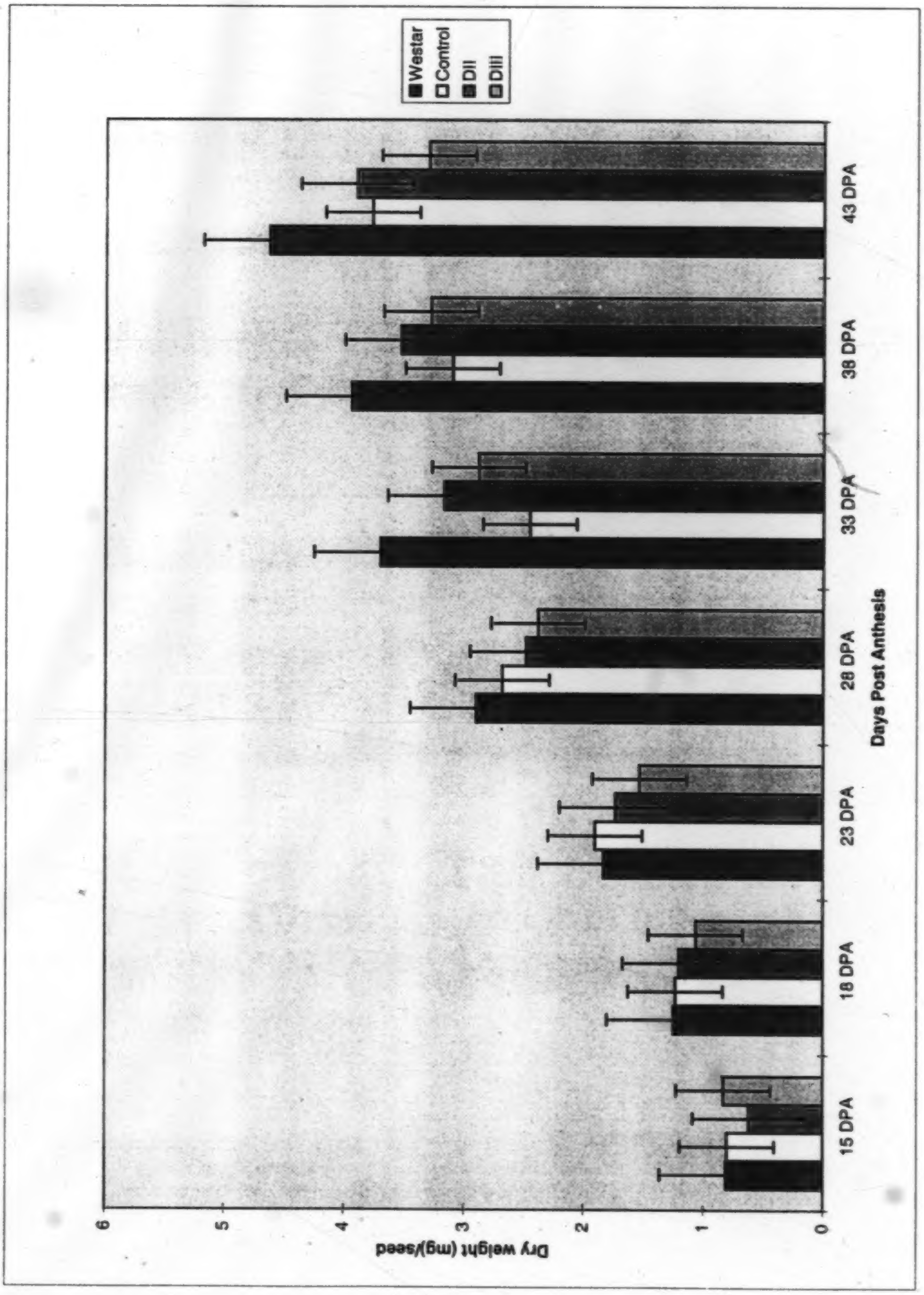

Dry weight (mg)/seed
0
1
2
3
4
5
6
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
■ Westar
□ Control
▨ DII
▨ DIII

FIG. 24: Effect of antisense cab on total protein accumulation per seed in transgenic *Brassica napus*. Total acetone precipitable protein was extracted from a known number of seed and resuspended in known volumes of protein buffer. Using calculated protein concentrations, volumes of protein buffer and number of seed used for extractions, total protein per seed was calculated. Values represent the mean ± S.E., n=3.

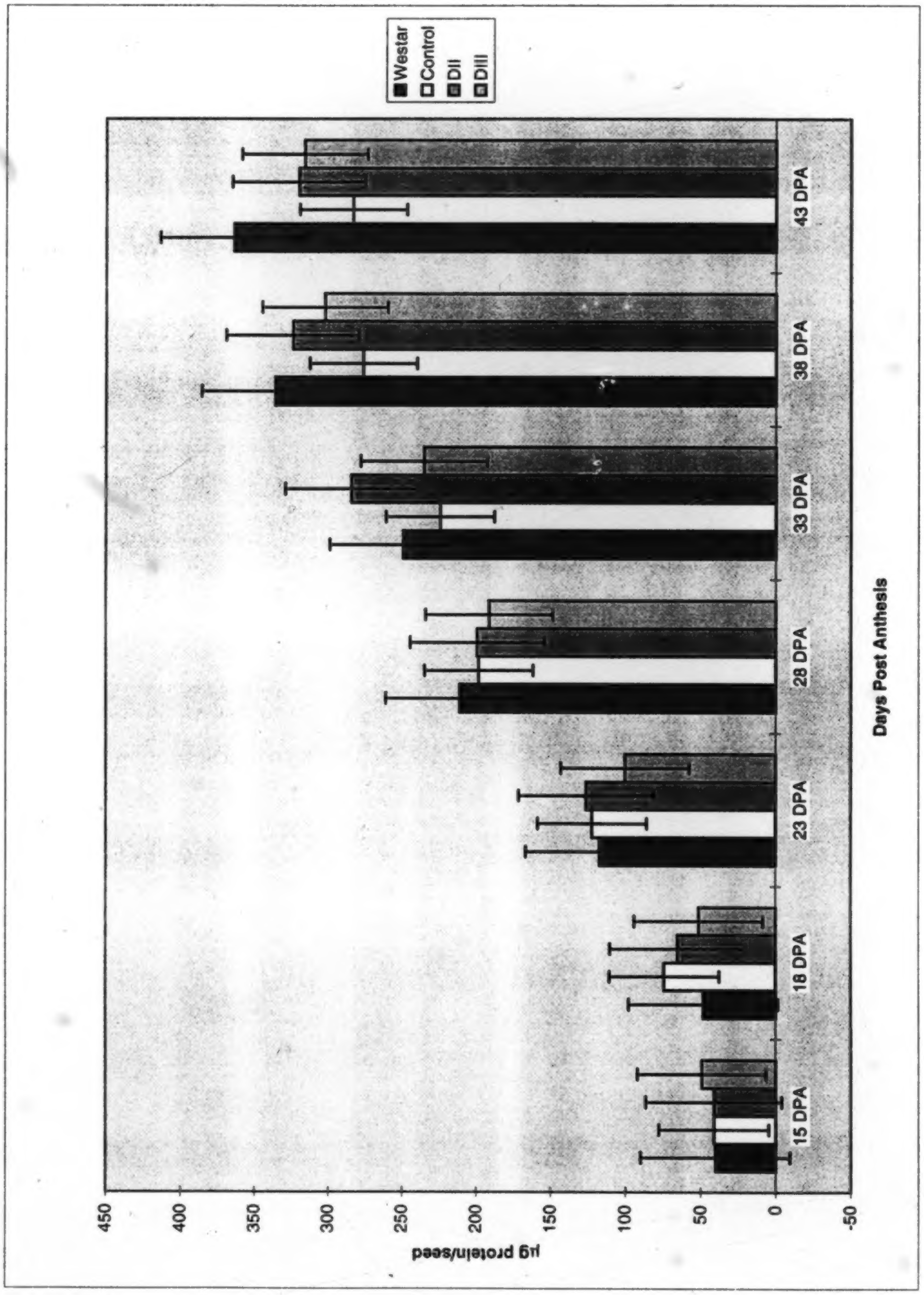
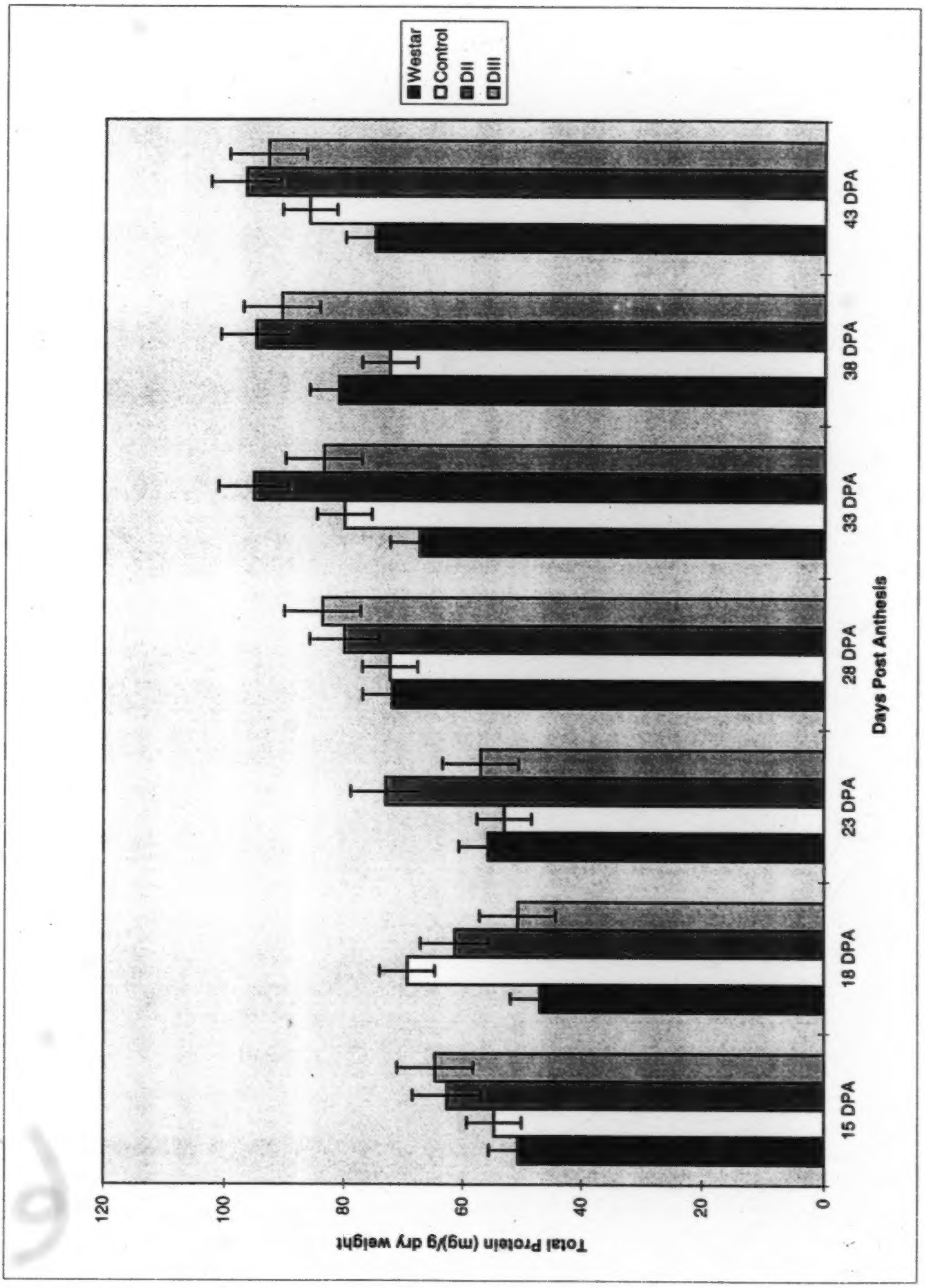

μg protein/seed
450
400
350
300
250
200
150
100
50
0
-50
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
■ Westar
□ Control
▨ DII
▨ DIII

FIG. 25: Effect of antisense cab on total protein accumulation per gram dry seed weight in transgenic *Brassica napus.* Total acetone precipitable protein was extracted from a known number of seed and resuspended in known volumes of protein buffer. Using calculated protein concentrations, volumes of protein buffer, average dry seed weight and number of seeds used for extractions, total protein per gram dry weight was calculated. Values represent the mean ± S.E., n=3.


Total Protein (mg)/g dry weight
0
20
40
60
80
100
120
15 DPA
18 DPA
23 DPA
28 DPA
33 DPA
38 DPA
43 DPA
Days Post Anthesis
■ Westar
□ Control
▨ DII
▨ DIII

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5: DISCUSSION

In recent years, the relationship between Cab protein and chlorophyll has received increasing attention from the scientific community. It has become generally accepted that Cab proteins degrade in the absence of chlorophyll (Cuming and Bennett, 1981). The converse relationship whereby chlorophyll accumulation is dependent upon appropriate Cab protein folding also appears to be true (Plumley and Schmidt, 1995). With the codependent relationship of Cab proteins and chlorophyll, it is logical to hypothesize that a reduction in Cab protein accumulation in seed of *Brassica napus* should induce a reduction in levels of chlorophyll accumulation. This hypothesis forms the basis of the research conducted in this project.

Antisense RNA has been used successfully in many circumstances to reduce the accumulation of certain proteins (Knutson et al., 1992, Kramer and Redenbaugh, 1994). For example, levels of the polygalacturonase enzyme were decreased in the case of the FLAV'R SAV'R tomato (Kramer and Redenbaugh, 1994) and stearoyl-ACP desaturase accumulation was reduced in developing *Brassica napus* embryos using antisense technology (Knutzon et al., 1992). The accuracy of targeting specific genes/gene families, the benefits of dealing with single gene effects and the successful application of antisense RNA in past research were several factors that contributed to the decision to use an antisense type I cab of PSII to attempt to reduce Cab accumulation in *Brassica napus* seed.

The intent of the present study was to use antisense cab to reduce steady-state mRNA levels, and thus reduce the production of Cab proteins. Other studies have also used antisense cab to reduce cab mRNA levels. Flachmann and Kuhlbrandt (1995) reduced cab mRNA to 5% through the use of antisense cab. At this level of suppression, they demonstrated normal levels of Cab and chlorophyll. However, in the course of their experiments, they also found plants with reduced pigment content, but chose not to study them (pers. comm.).

The results of Flachmann and Kuhlbrandt (1995) point to a common problem in antisense technology and that is the inexplicably variable results that are obtained. The variety of phenotypes that can be generated from transformation using the same antisense construct gives an indication of the variety of effects that antisense can cause. Stockhaus found that antisense transgenic plants expressed an antisense phenotype but expressed no reduction in target RNA (Stockhaus et al., 1990). Antisense studies by van der Krol et al. (1988) revealed the development of a variety of pigmentation patterns in *Petunia hybrida* petals resulting from introduction of a single antisense construct. In the present study, differences in suppression were noted between DII and DIII. Both chlorophyll and Cab protein levels measured on a per seed basis were lower in DIII relative to DII. While this may reflect the lack of homozygosity of DII, it may also arise as a result of insertion site in the genome, causing a very subtle antisense phenotype in DII. Thus, it is very difficult to predict or explain the results of antisense gene expression.

The choice of promoter dramatically influences the effectiveness of the antisense gene in reducing protein accumulation. In the present study, the gNa promoter was selected, as it is reported to begin transcription at 18 DPA and peak at 36 DPA (Blundy et al., 1991). According to previous work (Johnson-Flanagan, unpublished), chlorophyll content in canola seed peaks at approximately 28 DPA. Similarly, chlorophyll content in seed from the present study peaks at 28-33 DPA, depending upon the line. However, recent work by a collaborator has shown that changes in cab message precede chlorophyll changes (Green, pers. comm.). Thus, while the timing of napin promoter activity appears to be adequate for reducing Cab accumulation in the present study, an earlier promoter, such as phaseolin, may prove to be more effective.

The two transformant *Brassica napus* lines, DII and DIII, were subjected to southern blot analysis to determine copy number of the pNABI construct. The Southern blots revealed that each transgenic contained a single copy of the antisense cab construct. Gus analyses of the T2 generations showed that while DIII is homozygous for the antisense construct, DII does not appear to carry pNABI in the homozygous state. Both lines were used for subsequent analysis.

Examination of the mean chlorophyll levels for the four plant lines revealed that DIII has significantly less chlorophyll than does Westar from 33 DPA onward and has the lowest mean chlorophyll content of the four lines. DIII also contains lower levels of chlorophyll than the other three lines at 33 and 38 DPA. Chlorophyll levels were also significantly reduced in DII relative to Westar at 33 and 38 DPA, but DII failed to show a significant overall reduction in mean chlorophyll content relative to Westar.

In order to determine whether the reduced chlorophyll seen in DIII is the result of antisense, a number of approaches can be taken. These include Cab westerns, chlorophyll a/b ratios and northern blotting. The present study addressed the first two approaches, and the third, northern blotting, is ongoing in the lab.

In the present study, Cab protein accumulation was examined during seed development using western blots probed with α-CPIa antibody (White and Green, 1987a). One set of western blots examined Cab protein accumulation during seed development in each plant line. The second set of western blots compared Cab accumulation among the four plant lines at each of the seven examined DPA. Several western blots were subjected to densitometry in order to assign values to the amount of Cab protein in each lane. The results of the comparative western blots indicated that Cab accumulation was reduced in DIII compared to Westar at 28, 33 and 38 DPA with the differences being significant at 28 DPA. At 33 and 38 DPA, the mean Cab accumulation for Westar is consistently higher than DIII by at least 78% of the least significant difference. These differences between Westar and DIII in terms of Cab protein accumulation may be indicative of an antisense cab effect experienced by the DIII line. A larger data set may allow the differences in Cab accumulation seen between Westar and DIII at 33-38 DPA to be recognized as significant.

Flachmann and Kuhlbrandt (1995) examined Cab protein accumulation in antisense cab transgenic tobacco leaves with the use of protein gel blot analysis and immunodetection. They found that only LHCII monomers reacted with the antibody and quantitation using immunostaining was difficult. Their research suggested that immunodetection was not a simple and reliable method of analyzing Cab accumulation, particularly if quantitative results are desired. On the other hand, Green has been able to very effectively measure Cab proteins using protein gels and western blots (White and Green, 1987a,b). The difference may arise because Flachmann and Kuhlbrandt were able to identify only monomers of LHCII in their study, whereas Green was able to identify both monomeric and oligomeric forms of LHCII using the α-CPIa antibody (White and Green, 1987a). As well, Green was able to identify a PSI antenna chlorophyll/protein complex containing 4 polypeptides and CP29. Even in the case of a chlorophyll-b-less barley mutant, polypeptides belonging to each of the three major chlorophyll a and b protein complexes were identified using an antibody to spinach LHCII (White and Green, 1987b). In addition to being able to identify numerous different Cab proteins, Green has used westerns repeatedly to detect changes in levels of accumulation of different Cab polypeptides in leaves of *Hordeum vulgare* (White and Green, 1987a,b, 1988). As the present study employed the α-CPIa antibody of White and Green (1987), quantitation should be possible.

In the western blots from this study, Cab polypeptides from the light harvesting complexes, LHCII and LHCI, are plainly visible. However, although LHCIIb (26 Kd) is reported to be the most prevalent chlorophyll a/b binding protein in the chloroplast (Peter and Thornber, 1991), examination of band intensity on the comparative western blots revealed that the band for LHCIIa (29 Kd) was of much greater intensity than that for LHCIIb. These unexpected alterations in band intensity may simply be a result of the particular antibody used. Although the use of α-CPIa has proven to be an effective antibody, allowing quantitation of Cab proteins from Western blots, the use of an LHCII specific antibody, such as CP2 (1987a), may prove to provide a more accurate assessment of changes in LHCII polypeptides, particularly LHCIIb, in the transgenics relative to Westar. AS it was an antisense version of a type I cab gene encoding the LHCIIb polypeptide used in the pNABI construct, it may be prudent to examine Cab polypeptide accumulation with specific reference to the LHCIIb polypeptide. It must be recognized that sequence similarities within the cab gene family may also allow repression of more than just the target gene by the antisense cab construct, and consequently, the accumulation of many different Cab polypeptides may be affected and should, therefore, also be monitored.

In order to measure changes in Cab accumulation in leaves, Flachmann and Kuhlbrandt (1995) relied, in part, upon chlorophyll a/b ratios. LHCII complexes bind most of the chlorophyll b in plants making the ratio of chlorophyll a to chlorophyll b, in the opinion of Kuhlbrandt and Flachmann, an excellent indicator of changes in the amount of Cab proteins. The limitation of this system is that there are at least 3 different chlorophyll/protein complexes which bind chlorophyll b;

CP29, LHCII and LHCI (White and Green, 1988). Changes in chlorophyll a/b ratios do indicate changes in Cab protein accumulation but do not specify which of the 3 chlorophyll/protein complexes are being affected (Bennett, 1981).

In the present study, little alteration in the chlorophyll a/b ratios was detected. This may result from the reduced light levels reaching the seed. In comparison to leaf tissue, seed receives only about 10% of the light energy that leaves receive (Green, per. comm.). This reduced light is a result of filtration through the silique. Chow and Anderson indicated that plants exposed for long periods to a particular light condition are able to adapt by altering the arrangement of the thylakoid, varying the PSI/PSII ratio or changing the amount of light harvesting pigments serving each photosystem (Chow and Anderson, 1987a,b). During development, light penetration would be expected to decrease as the silique and seed coat collectively become more dense. It is possible that the developing canola seed contain increasing quantities of light harvesting antenna to maximize the use of the light. Light harvesting proteins, such as LHCII and LHCI, bind chlorophyll b. The more antenna proteins present, the higher the levels of chlorophyll b. Thus, such a response could make it difficult to detect subtle changes in the chlorophyll a/b ratio.

In the present study chlorophyll a/b ratios fluctuated in seed between 2-2.5 during development. In comparison, chlorophyll a/b ratios from tobacco leaves fluctuated around 3.6 (Flachmann and Kuhlbrandt, 1995). These results suggest that as a result of low irradiance, seed does contain higher levels of chlorophyll b, higher levels of Cab protein and consequently, lower chlorophyll a/b ratios relative to leaves.

Northern blot analysis of sense cab RNA has been examined during development in seed of Control and T2 seed of DIII (Politeski Morissette et al., unpublished). Although accumulation of sense RNA appears to follow a similar pattern in both lines up to 33 DPA, difference in RNA levels become obvious thereafter. At 38 DPA, sense RNA levels were noticeably lower in DIII than in the Control seed. By 43 DPA, sense cab RNA is undetectable in DIII. These observations are consistent with the expected peak activity of the gNa napin promoter and indicate that the antisense is reducing cab RNA steady-state levels in the DIII transgenic. This supports the idea that reduction in Cab and chlorophyll are a result of antisense cab.

Three results from the present study require further investigation. First, Cab suppression of chlorophyll at 33 DPA is inconsistent with a lack of cab mRNA suppression at that DPA. This probably reflects the fact that the mRNA results are preliminary in nature. Second, Cab western blots fail to demonstrate significant differences in Cab levels between Westar and DIII at 43 DPA. This is probably caused by low overall Cab levels and should not detract from the observation that levels are reduced from 28-38 DPA. Third, Cab protein suppression in DIII at 28 DPA is not reflected in suppressed chlorophyll or cab mRNA levels. Again, this may reflect the preliminary nature of our RNA results.

The present study provides insight into the relationship between chlorophyll and Cab. Although Cab and chlorophyll are highly correlated, there are specific examples showing discrepancies between chlorophyll and Cab protein levels in both the developmental and comparative western blots. In developmental western blots, chlorophyll and Cab protein levels in Westar and Control did not appear to be following the same trends. In a comparative western, at 33 DPA, the chlorophyll levels for Westar far exceeded the chlorophyll levels for the other 3 lines, while the Cab levels for Westar, Control and DII were very similar. The high Cab levels found in DII may be explained by the unpredictable nature of many antisense transgenics. The high Cab levels found in Control are unexplainable at this time. However, one may speculate that the strange behaviour of the Control may derive from the origins of the line. The tissue culture control line was derived from a single regenerated *Brassica napus* cv. Westar shoot. If the regenerating shoot was subject to random mutation during development, all of the plant derived from the original shoot may also contain the same disturbance. Such a random mutation may induce strange developmental effects, such as those seen in the Control line in this study. The use of a population of independently regenerated *Brassica napus* plants as a tissue culture control may have prevented the unexpected phenotypes seen in the Control line in this study.

Recent research has revealed that the relationship between Cab and chlorophyll levels may not be straightforward. In experiments conducted by Plumley and Schmidt (1995), chlorophyll b deficient mutants were examined for post-translational control of LHCII accumulation and accumulation of light harvesting pigments. One of these mutants, PS2.1, is believed to carry its defect as problematic translocation through the stroma and/or integration of LHCPII into the thylakoid (Plumley and Schmidt, 1995). It was found that with the reduced accumulation of LHCII, chlorophyll and xanthophylls were recovered only to the extent that they were incorporated into PSI/PSII complexes and minor LHC. Each pigment moiety appears to have only been synthesized to the extent to which it was needed. Additional experiments involved the use of the GE2.27 mutant, which appeared to be defective at one of the latter stages of chlorophyllide b biosynthesis. GE2.27, although chlorophyll b deficient, accumulated wild type levels of all LHCP. However, LHCPII, which appeared to assemble low levels of chlorophyll b, was found to be at intermediate stages of chlorophyll/protein complex formation. Revertants of GE2.27 contained varying gradations of chlorophyll b deficiencies. A positive relationship was found between levels of chlorophyll b and completely integrated LHCPII. These results suggest that there is a dependency of LHCPII upon chlorophyll b to allow for complete integration into thylakoid membranes.

On the other hand , studies involving the *chlorina* f2 barley mutant suggest a less obvious relationship between chlorophyll and Cab. The *chlorina* f2 barley mutant, deficient in chlorophyll b, has been studied extensively in terms of its LHC content (White and Green, 1987b, 1988, Harrison et al., 1993). A study by Krol et al. (1995) determined that the LHCIIb proteins associated with PSII were severely diminished compared to wild type barley, as would be expected when there is a chlorophyll b deficiency. However, LHCIa proteins associated with PSI did not significantly differ from wild type, despite the chlorophyll b deficiency. The levels of accumulation of particular Cab species did not appear to correlate with chlorophyll b levels equally. This study suggested that LHC polypeptides may be stable in some situations despite an absence of pigments (Krol et al., 1995).

Deleterious effects on the phenotypes are a major concern whenever the genetic makeup of a plant is altered. In the present study, the pattern of seed development was monitored by measuring changes in moisture content, weight, and protein content throughout development. No appreciable differences between the four plant lines were measured up to 38 DPA.

It appears that reduced chlorophyll levels do not appear to substantially influence seed size up to 38 DPA. This result is not surprising. In studies conducted by Flinn and Pate (1970), the photosynthetic contribution of seed to seed development was analyzed in *Pisum arvense*. Seed containing wild type levels of chlorophyll were excised from pods and exposed to full sunlight. These peas were found to fix only 80% of the carbon lost through normal respiration, although they were exposed to higher levels of sunlight than found within pea pods. Since peas and canola show similarities in the sources of photosynthates used for seed development (Flinn and Pate, 1973, Rood et al., 1984) the photosynthetic ability of peas and canola seed may also be similar. Thus, it would stand to reason that it is quite unlikely that reduced chlorophyll levels and reduced Cab levels in *Brassica napus* seed should substantially influence the dry weight of the transgenic seed. Although slight reductions in the seed dry weight were seen in DIII seed at 43 DPA, it has not been determined whether or not the loss of dry weight was a result of reduced chlorophyll levels. This reduction in dry seed weight may prove to have negligible economic importance as many canola producers are willing to accept the possibility of a slightly reduced yield if the occurrence of green seed can be reduced.

The results from the present study suggest that antisense Cab is less deleterious than other reported approaches. Attempts have been made by other researchers to lower chlorophyll levels in plants by inserting an antisense version of the gene encoding the C5 pathway enzyme, glutamate-1-semialdehyde aminotransferase (GSA-AT) (Hofgen et al., 1994). This enzyme is essential for the formation of ALA, the precursor of tetrapyrroles such as chlorophyll and heme. The antisense gene repressed expression of GSA-AT, which in turn reduced the formation of ALA (Hofgen et al., 1994). It is believed that reduced levels of tetrapyrroles, as a result of reduced ALA, led to severe plant damage.

The results of the present study indicate that chlorophyll levels in the DIII transgenic are significantly lower than chlorophyll levels in Westar. These results may have important repercussions in the canola industry. Green seed is a serious problem for canola farmers, particularly in areas of northern Alberta where early sub-lethal freezing temperatures are fairly common. A difference of only one percent green seed can determine whether a particular canola harvest is classified as Canola No.1, a superior grade canola, or Canola No. 2, a mid-grade canola. As such, even a small reduction in seed chlorophyll levels may prove to be of significant benefit to canola producers.

In conclusion, the goal of this project was to reduce chlorophyll accumulation in seed of *Brassica napus* using antisense technology. Analysis of cab RNA levels, chlorophyll levels, and Cab protein accumulation (Politeski Morissette et al., unpublished) in the seed of the four examined plant lines revealed successful application of antisense technology in DIII. Seed of DIII were found to contain reduced Cab protein levels and chlorophyll levels compared to seed of Westar. DIII seed also contained reduced cab sense RNA levels compared to Control. The use of an earlier promoter may enhance the differences seen in chlorophyll levels, Cab protein levels and cab RNA levels. Compared with other methods of chlorophyll reduction, such as reducing the expression of genes responsible for the production of ALA (Hofgen et al., 1994), using antisense technology to reduce Cab protein accumulation appears to have negligible deleterious effects on seed protein and moisture content.

The chlorophyll reductions seen in this study in the DIII transgenic are expected to be enhanced after frost. Frost induces a number of responses, including dessication (Johnson-Flanagan et al., 1991) and increases in ABA (Johnson-Flanagan et al., 1992). As ABA and increased osmotic potential (a condition associated with desiccation) induce napin expression (Crouch et al., 1985) and the antisense cab construct is driven by a napin promoter, a sub-lethal frost should strongly induce transcription of the antisense cab gene. This in turn would be expected to reduce the amount of Cab protein incorporated into thylakoid membranes, and reduce the chlorophyll levels. In the absence of antisense, we would expect to see an increase in chlorophyll accumulation since sub-lethal frost also induces renewed chlorophyll synthesis (Johnson-Flanagan et al., 1990). As such, any ameliorating effect of the antisense cab should be greater after a frost as compared with the control conditions in the present study.

6: CONCLUSION

Green seed is a serious problem for the canola industry in western Canada. As little as 6% green seed can seriously reduce the value of a canola crop. In order to help alleviate the green seed problem, this study has attempted to reduce chlorophyll accumulation in seed of transgenic *Brassica napus* cv. Westar.

The antisense version of a type I cab gene of Photosystem II was inserted into the genome. Examination of T2 seed of the transgenic plants revealed that chlorophyll and Cab protein levels in the DIII transgenic appeared to be lower than chlorophyll and Cab levels in Westar and Control. Preliminary examination of the transgenic seed failed to show any deleterious effects in terms of seed weight, seed development, measured as moisture content, or protein content. However, further investigations of various aspects of seed oil content, storage protein accumulation and steady state mRNA levels remain to be conducted. Initial results suggest that using antisense technology to reduce the accumulation of Cab proteins is an effective method for reducing chlorophyll accumulation in seed of *Brassica napus*.

7: FUTURE PROSPECTS

In the present study, a significant reduction in chlorophyll levels of up to 48% was obtained in DIII compared to Westar. While my work has proven the technology, a great deal of research remains to be conducted to determine the effects of antisense cab gene expression on seed development. RNA analysis, through the use of northern blots, are needed and are ongoing. Lipid and storage protein analyses are required to determine if seed storage products have been altered as a result of antisense cab expression. It may also prove to be beneficial to conduct a more detailed examination of seed pigments during development. A more detailed analysis may reveal how different pigment species, such as pheophytins, are affected by expression of the antisense cab gene. The resulting information may reveal changes in pigment degradation as a result of the expression of antisense cab genes. It would also be imperative to examine chlorophyll, RNA, lipid, storage proteins, Cab levels and perform detailed pigment analysis after developing seed have been exposed to a sub-lethal frost. Since the antisense construct was designed for enhanced expression following a frost, it is important to examine the effects of the antisense construct on the seed after exposure to sub-lethal frost. In the current study, the antisense cab gene is driven by a napin promoter. A variety of promoters, however, could be used in other experiments to drive the tissue specific expression of the antisense cab gene. An earlier promoter, such as phaseolin, may prove to reduce Cab accumulation more than seen in this study. It may also prove interesting to examine the effect of abalogues (ABA analogues) (Abrams, pers. comm.) on ABA responsive promoters which could be used to drive the antisense construct.

8: REFERENCES

Abraham, V., deMan J.M., (1986) Hydrogenation of canola oil as affected by chlorophyll. JAOCS 63: 1185-1188

Alfonso, M., et al., (1994) Core antenna complexes, CP43 and CP47, of higher plant photosystem II: Spectral properties, pigment stoichiometry, and amino acid composition. Biochemistry 33: 10494-500

Amir-Shapira, D., Goldschmidt, E. Altman, A., (1987) Chlorophyll catabolism in senescing plant tissues: *In vivo* breakdown intermediates suggest different degradative pathways for citrus fruit and parsley leaves. PNAS 84: 1901-1905

Anderson, J., (1992) Cytochrome b_6f complex: Dynamic molecular organization, function and acclimation. Photosynthesis Research 34: 341-357

Apel, K., (1978) Phytochrome-induced appearance of mRNA activity for the apoprotein of the light-harvesting chlorophyll a/b protein of barley. Eur. J. Biochem. 97: 183-188

Barton, K., Chilton, M., (1983) *Agrobacterium* Ti plasmids as vectors for plant genetic engineering. Methods in Enzymology 101: 527-539

Bassi, R., Rigoni, R., and Giacometti, GM., (1990) Chlorophyll binding proteins with antenna function in higher plants and green algae. Photochem. Photobiol. 52: 1187-1206

Bassi, R., Rigoni, R., Barbarto, R., and Giacometti G., (1988) Light-harvesting chlorophyll a/b proteins (LHCII) populations in phosphorylated membranes. Biochim. Biophys. Acta 936: 29-38

Baumgartner, B., Rapp, J., Mullet, J., (1993) Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (*Hordeum vulgare*) chloroplast development. Plant Physiol. 101: 781-791

Beale, SI., Weinstein, JD., (1990) Tetrapyrrole metabolism in photosynthetic organisms. In HA Dailey, Ed, Biosynthesis of Heme and Chlorophylls. McGraw-Hill, New York pp. 287-391

Beck, E., Ludwig, G., Averswald, E., Reiss, B., Schaller, H., (1982) Nucleotide sequence and exact location of the neomycin phosphotransferase gene from transposon Tn5. Gene 19: 327-336.

Bennett, J., (1980) Chloroplast phosphoproteins: Evidence for a thylakoid-bound phosphoprotein phosphatase. Eur. J. Biochem. 104: 85-89

Bennett, J., (1981) Biosynthesis of the light-harvesting chlorophyll a/b protein. Eur. J. Biochem. 118: 61-70

Bennett, J., (1983) Regulation of photosynthesis by reversible phosphorylation of the light-harvesting chlorophyll a/b protein. Biochem. J. 212: 1-13

Bevan, M., (1984) Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res. 12: 8711-8721

Bhaya D., Castelfranco, P., (1985) Chlorophyll biosynthesis and assembly into chlorophyll-protein complexes in isolated developing chloroplasts. PNAS 82: 5370-5374

Biggins, J., (1982) Thylakoid conformational changes accompanying membrane protein phosphorylation. Biochim. Biophys. Acta 679: 479-482

Blundy, K., Blundy, M., Crouch, M., (1991) Differential expression of members of the napin storage protein gene family during embryogenesis in *Brassica napus*. Plant Mol. Biol. 17: 1099-1104

Boekema, E., Wynn, R., Malkin, R., (1990) The structure of spinach photosystem I studied by electron microscopy. Biochim. Biophys. Acta 1017: 49-56

Burgi, R., Suter, F., Zuber, H., (1987) Arrangement of the light-harvesting chlorophyll a/b protein complex in the thylakoid membrane. Biochim. Biophys. Acta 890: 346-351

Butler, P., Kuhlbrandt, W.,(1988) Determination of the aggregate size in detergent solution of the light-harvesting chlorophyll a/b-protein complex from chloroplast membranes. PNAS USA. 85: 3797-3801

Canola Council of Canada (1991) Canada's Canola. Canola Council of Canada, Winnipeg, Manitoba. pp. 1-16

Cangelosi, G., Best, E., Martinetti, G., and Nester, E., (1991) Genetic analysis of *Agrobacterium*. Methods in Enzymology 204: 384-397

Caplan, L., Herrera-Estrella, L., Inze, D., Van Haute, E., Van Montagu, M., Schell, J., Zambryski, P., (1983) Introduction of genetic material into plant cells. Science 222: 815-821

Chang, Y., Walling, L., (1991) Abscisic acid negatively regulates expression of chlorophyll a/b binding protein genes during soybean embryogeny. Plant Physiol. 97: 1260-1264

Chow, W., Anderson, J., (1987a) Photosynthetic responses of *Pisum sativum* to an increase in irradiance during growth I. Photosynthetic activities. Aust. J. Plant Physiol. 14: 1-8

Chow, W., Anderson, J., (1987b) Photosynthetic responses of *Pisum sativum* to an increase in irradiance during growth II. Thylakoid membrane components. Aust. J. Plant Physiol. 14: 9-19

Chow, W., Adamson, H., Anderson, J., (1991) Photosynthetic acclimation of *Tradescantia albiflora* to growth irradiance: Lack of adjustment of light-harvesting components and its consequences. Physiol. Plant. 81: 175-182

Crouch, M., et al., (1985) Storage protein mRNA levels can be regulated by abscisic acid in *Brassica* embryos. In L van Vloten Doting, GSP Groot, TC Hall, eds, Molecular Form and Function of the Plant Genome, Plenum, New York, pp 555-566

Coleman, J., Hirashima, A., Inokuchi, Y., Green, P., and Inouye, M., (1985) A novel immune system against bacteriophage infection using complementary RNA (micRNA). Nature 314: 601-603

Cooper, P., and Ort, D., (1987) The effect of chilling on the production of photosystem II light-harvesting chlorophyll a/b binding protein in tomato. In Plant Gene Systems and Their Biology. Alan R. Liss, Inc. pages 353-362

Crowley, T., et al., (1985) Phenocopy of discoidin 1-MINUS mutants by antisense transformation in *Dictyostelium*. Cell 43: 633-641

Cuming, A., and Bennett, J., (1981) Biosynthesis of the light-harvesting chlorophyll a/b protein. Eur. J. Biochem. 118: 71-80

Dahlen, J., (1973) Chlorophyll content monitoring of Swedish rapeseed and its significance in oil quality. JAOCS 50: 312A-327A

DeClercq, D., Daun, J., and Tipples, K., (1994) Quality of Canadian Canola and Flaxseed Cargoes 1993-94 Crop year. Canadian Grain Commission, Winnipeg, Manitoba. pp. 2-7

DeClercq, D., Daun, J., and Tipples, K., (1995) Quality of Western Canadian Canola 1995. Canadian Grain Commission, Winnipeg, Manitoba. pp. 3-8

Dennis, D., and Turpin, D., (1990) Plant Physiology, Biochemistry and Molecular Biology. Longman Scientific and Technical, Essex, England. pp, 9-10, 198-208

Dellaporta, S., Wood, J., and Hicks, J., (1983) A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19-21

DePetter, E., van, Wiemeersch,L., Rethy, R., Dedonder, A., Fredericq, H., Greef, J., (1988) Fluence-response curves and action spectra for the very low fluence and the low fluence response for the induction of Kalanchoe seed germination. Plant Physiol. 88: 276-283

Diosady, L., (1991) Chlorophyll removal from canola oil - ion exchange. In 9th Progress Report - Research on Canola Seed Oil and Meal. Canola Council of Canada. Winnipeg, Manitoba. pp. 441-446

Dreyfuss, B., and Thornber, J., (1994) Assembly of the light-harvesting complexes (LHCs) of Photosystem II. Plant Physiol. 106: 829-839

Dunn, S., (1986) Effect of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal. Biochem. 157: 144-153

Dunsmuir, P., (1985) The petunia chlorophyll a/b binding protein genes: A comparison of cab genes from different gene families. Nucl. Acids Res. 13: 2503-2518

Flores, S., and Tobin, E., (1988) Cytokinin modulation of LHCP mRNA levels: The involvement of post-transcriptional regulation. Plant Mol. Biol. 11: 409- 415

Falconet, D., White, M., Fristensky, B., Dobres, M., Thompson, W., (1991) Nucleitide sequence of cab-215, a type II gene encoding a photosystem II chlorophyll a/b-binding protein in *Pisum.* Plant Mol. Biol. 17: 135-139

Falconet, D., Godon, C., White, M.J., and Thompson, W.F., (1993) Sequence of lhcb3*1, a gene encoding a photosystem II chlorophyll a/b-binding protein in *Pisum.* Biochim. Biophys. Acta 1173: 333-336

Flachmann, R., and Kuhlbrandt, W., (1995) Accumulation of plant antenna complexes is regulated by post-transcriptional mechanisms in tobacco. The Plant Cell 7: 149-160

Flinn, A., (1969) A nutritional study of fruit maturation in *Pisum arvense* L. Ph.D. thesis, The Queen's University, Belfast

Flinn, A., and Pate, J., (1970) A quantitative study of carbon transfer from pod and subtending leaf to the ripening seeds of the field pea (*Pisum arvense* L.) Journal of Experimental Botany 21: 71-82

Freyman, S., Charnetski, W., and Crookston, R., (1973) Role of leaves in the formation of seed in rape. Can. J. Plant Sci. 53: 693-694

Garfinkel, D., and Nester, E., (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. 144: 732-743

Garfinkel, D., Simpson, R., Ream, L., White, F., Gordon, M., and Nester, E., (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site directed mutagenesis. Cell 27: 143-153

Golbeck, J., (1992) Structure and function of Photosystem I. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 293-324

Grafflage, S., and Krause, G., (1986) Simulation of in situ freezing damage of the photosynthetic apparatus by freezing in vitro of thylakoids suspended in complex media. Planta 168: 67-76

Green, B., (1988) The chlorophyll-protein complexes of higher plant photosynthetic membranes or Just What Green Band is That? Photosynthesis Research 15:3-32

Green, B., and Durnford, D., (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 685-714

Green, B., and Johnson-Flanagan, A., (1993) Role of proteases and seed dessication rate in the green seed problem in canola. NSERC Strategic Grant Application

Green, B., Sen, D., Aebersold, R., and Pichersky, E., (1992) Identification of the polypeptides of the major light-harvesting complex of Photosystem II (LHCII) with their genes in tomato. FEBS Lett. 305: 18-22

Green, B., (1995) Rapeseed Congress

Harrison, M., Nemson, J., and Melis, A., (1993) Assembly and composition of the chlorophyll a/b light-harvesting complex of barley (*Hordeum vulgare* L.): Immunochemical analysis of chlorophyll b-less and chlorophyll b-deficient mutants. Photosynthesis Research 38: 141-151

Hauska, G., Hurt, E., Gabellini, N., and Lockau, W., (1983) Comparative aspects of quinol-cytochrome / plastocyanin oxidoreductases. Biochim. Biophys.. Acta 726: 97-133

Hendry, G., Houghton, J., and Brown, S., (1987) The degradation of chlorophyll - a biological enigma. New Phytol. 107: 255-302

Henningsen, KW., et al., (1993) Mutants at xantha and albina loci in relation to chloroplast biogenesis in barley (*Hordeum vulgare* L) Royal Can. Acad. Sci Lett. Biol. Skrifter

Hernalsteens, J., et al., (1980) The *Agrobacterium tumefaciens* Ti-plasmid as a host vector system for introducing foreign DNA in plant cells. Nature (London) 287: 654-656

Herrera-Estrella, L., Depicker, A., Van Montagu, M., and Schell, J., (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature (London) 303: 209-213

Hiatt, W., Kramer, M., and Sheehy, R., (1989) The application of antisense RNA technology to plants. In: Genetic Engineering Principles and Methods. Vol II. J.K. Setlow, ed. Plenum Press, New York. pp. 49-63

Hirsch, P., and Beringer, J., (1984) A physical map of pPH1JI and pJB4JI. Plasmid 12: 139-141

Hodgins, R., Pomeroy, K., Horak, A., and Johnson-Flanagan, A., (1989) Inhibition of photosynthetic activity in canola (*Brassica napus*) cv Westar seeds following freezing (abstract No. 158) Plant Physiol. 89: S-527

Hoekema, P., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A., (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179-180

Hofgen, R., Axelsen, K., Kannangara, G., Schuttke, I., Pohlenz, H., Willmitzer, L., Grimm, B., and von Wettstein, D., (1994) A visible marker for antisense mRNA expression in plants: Inhibition of chlorophyll synthesis with a glutamate-1-semialdehyde aminotransferase antisense gene. PNAS USA 91: 1726-173

Holbrook, L.A., van Rooijen, G.J.H., Wilen, R.W., Moloney, M.M., (1991) Oilbody proteins in microspore-derived embryos of *Brassica napus*. Plant Physiol. 97: 1051-1058

Holschuh, K., et al., (1984) Structure of the spinach chloroplast genes for the D2 and 44 Kd reaction-center proteins of Photosystem II and for tRNA^{ser} (UGA). Current Genetics 8: 597-606

Houch, C., Shintani, D., and Knauf, V., (1988) *Agrobacterium* as a gene transfer agent for plants. Frontiers Appl. Microbiol. 4:

Inouye, M., (1988) Antisense RNA: Its functions and applications in gene regulation - a review. Gene 72: 25-34

Internation Markets Bureau (1994) Oilseeds Sector Profile Markets and Industry Services Branch, Agriculture Canada, Ottawa, Ontario. pp. 2-15

Jansson, S., (1994) The light-harvesting chlorophyll a/b binding proteins. Biochim. Biophys. Acta. 1184: 1-19

Jefferson, R., (1985) Ph. D. Dissertation, University of Colorado, Boulder, Co

Jefferson, R., Burgess, S., and Hirsh, D., (1986) β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. Usa. 83: 8447-8451

Jefferson, R., Kavanagh, R., Bevan, M., (1987) EMBO J 6: 3901-3907

Johnson-Flanagan, A., (1992) Understanding and alleviating green seeds in spring canola: Impact of increased frost tolerance on degreening. The Alberta Agriculture Research Institute, Edmonton, Alberta. Project Report #: 88M012 pp. 8-17

Johnson-Flanagan, A., and Spencer, M., (1996) Chlorophyllase and peroxidase activity during degreening of maturing canola (*Brassica napus*) and mustard (*Brassica juncea*) seed. Physiol. Plant. 96: 1-7

Johnson-Flanagan, A., and McLachlan, G., (1990a) The role of chlorophyllase in degreening canola (*Brassica napus*) seeds and its activation by sublethal frost. Physiol. Plant. 80: 460-466

Johnson-Flanagan, A., and McLachlan, G., (1990b) Peroxidase-mediated chlorophyll bleaching in degreening canola (*Brassica napus*) seeds and its inhibition by sub-lethal freezing. Physiol. Plant. 80: 453-459

Johnson-Flanagan, A., and Thiagarajah, M., (1990) Degreening in canola (*Brassica napus*, cv. Westar) embryos under optimum conditions. J. Plant Physiol. 136: 180-186

Johnson-Flanagan, A., Singh, J., and Thiagarajah, M., (1990) The impact of sublethal freezing during maturation on pigment content in seeds of *Brassica napus*. J. Plant Physiol. 136: 385-390

Johnson-Flanagan, A., Thiagarajah, M., and Pomeroy, K., (1991) The impact of freezing during maturation on storage products in canola seeds. Physiol. Plant. 81: 301-308

Johnson-Flanagan, A., Huiwen, Z., Geng, X., Brown, D., Nykiforuk, C., and Singh, J., (1992) Frost, abscisic acid, and dessication hasten embryo development in *Brassica napus*. Plant Physiol. 99: 700-706

Johnson-Flanagan, A., and Singh, J., (1993) A method to study seed degreening using haploid embryos of *Brassica napus* cv. Topas. J. Plant Physiol. 141: 487-493

Joos, H., (1997) InVigor^{TM} Technology. Plant Biotech Institute Bulletin. National Research Council Canada. January Issue

Jorgensen, R., (1992) Silencing of plant genes by homologous transgenes. Agbiotech News Information 4: 265N-273N

Kannangara, C.G., et al., (1994) Enzymic and mechanistic studies on the conversion of glutamate to 5-aminolaevulinate. In The Biosynthesis of the Tetrapyrrole Pigments, Ciba Foundation Symposium 180. John Wiley and Sons, Chichester, England. pp. 3-20

Kellman, J.W., Pichersky, E., and Piechulla B., (1990) Analysis of the diurnal expression pattern of the tomato chlorophyll a/b binding protein genes. Influence of light and characterization of the gene family. Photochem. Photobiol. 52: 35-41

Kennedy, J., and Johnson-Flanagan, A., (1993) Molecular mechanisms causing the green seed problem in canola. Crop Molecular Biology and Biotechnology Workshop, Banff, Alberta

Kim, M., and Mullet, J., (1995) Identification of a sequence specific DNA binding factor acquired for transcription of the barley chloroplast blue light-responsive psbD-psbC promoter. Plant Cell 9: 1445-1457

Kim, S., and Wold, B., (1985) Stable reduction of thymidine kindase-activity in cells expressing high levels of antisense RNA. Cell 42: 129

Knutson, D., et al., (1992) Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. PNAS USA 89: 2624-2628

Kohno-Murase, J., Murase, M., Ichikawa, H., and Imamura, J., (1995) Improvement in the quality of seed storage protein by transformation of *Brassica napus* with an antisense gene for cruciferin. Theor. Appl. Genet. 91: 627-631

Kramer, M., and Redenbaugh, M., (1994) Commercialization of a tomato with an antisense polygalacturinase gene. The FLAV'R SAV'R tomato story. Euphytica 79: 293-297

Kridl, J., Knauf, V., and Thompson, G., (1992) In Control of Plant Gene Expression, ed. Verma, D.P.S. (CRC Boca Raton, Fl), Vol. 2

Kridl, J., McCarter, D., Rose, R., Scherer, D., Knutzon, D., Radke, S., and Knauf, V., (1991) Isolation and characterization of the expressed napin gene from *Brassica rapa*. Seed Science Research 1: 209-219

Krol, M., et al., (1995) Chlorophyll a/b-binding proteins, pigment conversions, and early light-induced proteins in a chlorophyll b-less mutant. Plant. Physiol. 107: 873-883

Kuhlbrandt, W., and Wang, D., (1991) Three-dimensional structure of plant light-harvesting complex determined by electron crystallography. Nature 350: 130-134

Laemmli, U., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lee. W., Tzen, J., Kridl, J., Radke, S., and Huang, A., (1991) Maize oleosin is correctly targeted to seed oil bodies in *Brassica napus* transformed with the maize oleosin gene. PNAS USA 88: 6181-6185

Lefebvre, D., (1990) Fundamentals of gene structure and control. In Plant Physiology, Biochemistry and Molecular Biology Ed. Dennis, D., and Turpin, D., Longman Scientific and Technical, Essex, England. p. 3-15

Lemmers, M., De Beuckeleer, M., Holsters, M., Zambryski, P., Depicker, A., Hernalsteens, J.P., Van Montagu, M., and Schell, J., (1980) Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumours. J. Mol. Biol. 144: 353-376

Lichtenstein, C., (1988) Anti-sense RNA as a tool to study plant gene expression. Nature 333: 801-802

Lindsten, A., Wiktorsson, B., Ryberg, J., Sundqvist, C., (1993) Chlorophyll synthetase activity is relocated from transforming prolamellar bodies to developing thylakoids during irradiation of dark green wheat. Physiol. Plant. 88: 29-36

Luthy, B., Martinoia, E., Matile, P., and Thomas, H., (1984) Thylakoid-associated chlorophyll oxidase: Distinction from lipoxygenase. Z. Pflanzenphysiol. 113: 423-434

Luthy, B., Thomas, H., and Matile, P., (1986) Linolenic acid dependent chlorophyll oxidase acivity: A property of photosystems I and II. J. Plant Physiol. 123: 203-209

Maloney, M.A., Huber, J.K., and Marks, D.B., (1989) Kinetics of chlorophyll accumulation and formation of chlorophyll-protein complexes during greening of *Chlamydomonas reinhardtii-Y*-1 at 38° C. Plant Physiol. 91: 1100-1106

Manitais, T., Frisch, E., and Sambrook, J., (1982) Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Matile, P., (1980) Catabolism of chlorophyll: Involvement of peroxidase? Z. Pflanzenphysiol. 99: 475-478

Matzke, M., and Matzke, A., (1995) How and why do plants inactivate homologous (trans)genes? Plant Physiol. 107: 679-685

McBride, K., and Summerfelt, K., (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol. Biol. 14: 269-276

Mehler, A., (1951) Studies of reactions of illuminated chloroplast. I. Mechanism of the reduction of oxygen and other reagents. Arch. Biochem. Biophys. 31: 65-77

Moloney, M., Walker, J., and Sharma, K., (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Reports 8: 238-242

Mullet, J.E., (1988) Chloroplast development and gene expression. Annu. Rev. Plant. Physiol. Mol. Biol. 39: 475-502

Mullet, J (1990) Molecular biology of photosynthesis in higher plants. In Plant Physiology, Biochemistry and Molecular Biology. Ed. Dennis, D. and Turpin, D. Longman Scientific and Technical. Essex, England. p. 198-211

Murai, N., and Kemp, J., (1982) Octopine synthase mRNA isolated from sunflower crown gall callus is homologous to the Ti-plasmid of *Agrobacterium tumefaciens*. PNAS USA 79: 86-90

Nagy, F., Fejes, E., Wehmeyer, B., Dallman, G., and Schafer, E., (1993) The circadian oscillator is regulated by a very low fluence response of phytochrome in wheat. PNAS USA. 90: 6290-6294

Nykiforuk, C., (1996) PhD thesis, University of Alberta, Edmonton, Alberta, Canada

Ohyama, A., Ito, H., Sato, T., Nishimura, S., Imai, T., and Hirai, M., (1995) Suppression of acid invertase activity by antisense RNA modifies the sugar composition of tomato fruit. Plant Cell Physiol. 36(2): 369-376

Okamoto, K., and Freundlich, M., (1987) Autogenous regulation of transcription of the crp operon by a divergent RNA transcript. In: Inouye, M. and Dudock, B. (Eds.) Molecular Biology of RNA: New Perspectives, Academic Press. Orlando, Fl, pp. 357-369

Parmenter, D., Boothe, J., van Rooijen, G., Yeung, E., Moloney, M., (1995) Production of biologically active hirudin in plant seeds (Abstract). Crop Molecular Biology and Biotechnology Workshop, Banff, Alberta

Peter, G., and Thornber, J., (1991) Biochemical composition and organization of high plant Photosystem II light-harvesting pigment-proteins. J. Biol. Chem. 266: 16745-16754

Petit, A., and Tempe, J., (1978) Isolation of *Agrobacterium* Ti-plasmid regulatory mutants. Mol. Gen. Genet. 167: 147

Piechulla, B., et al., (1991) Determination of steady-state mRNA levels in individual chlorophyll a/b binding protein genes of tomato cab gene family. Mol. Gen. Genet. 230: 413-422

Plumley, G., and Schmidt, G., (1995) Light-harvesting chlorophyll a/b complexes: Interdependent pigment synthesis and protein assembly. The Plant Cell 7: 689-704

Porra, RJ., et al., (1994) The derivation of the formyl-group oxygen of chlorophyll b in higher plants from molecular oxygen: Achievement of high enrichment of the 7-formyl-group oxygen from $^{18}O_2$ in greening maize leaves. Eur. J. Biochem. 219: 671-679

Prezelin, B., Nelson, N., (1990) The formation of ATP and reducing power in the light. In Plant Physiology, Biochemistry and Molecular Biology. Eds. Dennis, D. and Turpin, D. Longman Scientific and Technical. Essex, England. p. 212-223

Radke, S., Andrews, B.M., Moloney, M., Crouch, M.L., Kridl, J.C., and Knauf, V.C., (1988) Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: Developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Genet. 75: 685-694

Riesselmann, S., and Piecheulla, B., (1990) Effect of dark phases and temperature on the chlorophyll a/b binding protein mRNA level oscillation in tomato seedlings. Plant Mol. Biol. 14: 605-616

Rood, S., Major, D., and Charnetski, W., (1994) Seasonal changes in $^{14}CO_2$ assimilation and ^{14}C translocation in oilseed rape. Field Crops Research 8: 341-348

Rudiger, W., Benz, J., and Guthoff, C., (1980) Detection and partial characterization of activity of chlorophyll synthesis in etioplast membranes. Eur. J. Biochem. 109: 193-200

Sakaki, T., Kondo, R., and Sugahara, K., (1983) Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation: Role of active oxygens. Physiol. Plant. 59: 28-34

Sanders, P., Winter, J., Barnason, A., Rogers, S., Fraley, R., (1987) Comparison of the cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucl. Acids Res. 15: 1543-1588

Sandler, S., Stayton, M., Townsend, J., Ralston, M., Bedbrook, J., and Dunsmuir, P., (1988) Inhibition of gene expression in transformed plants by antisense RNA. Plant Mol. Biol. 11: 301-310

Satoh, K., (1993) Isolation and properties of the Photosystem II reaction center. In The Photosynthetic Reaction Center, ed J Deisenhofer, JR Norris, San Diego: Academic, 1: 289-318

Schoch, S., and Brown, J., (1987) The action of chlorophyllase on chlorophyll protein complexes. J. Plant Physiol. 126: 483-494

Schoch, S., and Vielwerth, F., (1983) Chlorophyll degradation in senescent tobacco cell culture (Nicotiana tabacum var. Samsun) Z. Pflanzenphysiol. Bd. 110: 309-317

Schoch, S. et al., (1984) 13^2-Hydroxychlorophyll a, the first product of the reaction of chlorophyll-oxidase. J. Plant Physiol. 115: 85-89

Seibert, M., (1993) Biochemical, biophysical and structural characterization of the isolated Photosystem II reaction center complex. In The Photosynthetic Reaction center, ed J Deisenhofer, JR Norris, San Diego: Academic, 1: 319-56

Simons, R., and Kleckner, N., (1983) Translational control of IS10 transposition. Cell 34: 683-691

Singh, R., and Chuaqui, C., (1991) Development of a continuous process to remove chlorophylls from canola oil. In 9th Progress Report - Research on Canola Seed Oil and Meal. Canola Council of Canada. Winnipeg, Manitoba. pp, 449-462

Sigrist, M., and Staehelin, L., (1994) Appearance of type 1, 2 and 3 light-harvesting complex II and light-harvesting complex I proteins during light-induced greening of barley (*Hordeum vulgare*) etioplasts. Plant Physiol. 104: 135-145

Stayton, MM., Block, M., Bedbrook, J., and Dunsmuir, P., (1986) A novel chlorophyll a/b binding (cab) gene from petunia which encodes the lower molecular weight Cab precursor protein. Nucl. Acids Res. 14: 9781-9796

Stockhaus, J., et al., (1990) Anti-sense RNA efficiently inhibits formation of the 10 kD polypeptide of Photosystem II in transgenic potato plants: Analysis of the role of the 10 kD protein. EMBO J. 9: 3013-3021

Tanaka, R., Yoshida, K., Nakayashiki, M., Tsuji, H., Inokuchi, H., and Tanaka, A., (1996) Differential expression of two hem A mRNAs encoding glutamyl-tRNA reductase proteins in greening cucumber seedling. Plant Physiol. 110: 1223-1230

Taylor, W.C., (1989) Regulatory interactions between nuclear and plastid genomes. Annu. Rev. Plant Physiol. Mol. Biol. 40: 39-59

Tayo, T., and Morgan, D., (1975) Quantitative analysis of the growth, development, and distribution of flowers and pods in oil seed rape (*Brassica napus* L.). J. Agric. Sci., Camb., 85: 103-110

Thornber, J., Peter, G., Morishige, D., Gomez, S., Anandan, S., Welty, B., Lee, A., Kerfeld, C., Takeuchi, T., and Preiss, S., (1993) Light harvesting in Photosystems I and II. Biochemical Society Transactions. 21: 15-18

Usuki, R., Endo, Y., and Kanada, T., (1984) Prooxidant activities of chlorophylls and pheophytins on the photooxidation of edible oil. Agric. Biol. Chem. 48: 991-994

Vaisey-Genser, M., and Eskin, N.A., (1982) Canola Oil, Properties and Performance. Canola Council of Canada, Winnipeg, Manitoba pp. 20-27

van der Krol, A., Mur, L., de Lange, P., Mol, J., and Stuitje, A., (1990) Inhibition of flower pigmentation by antisense CHS genes: Promoter and minimal sequence requirements for the antisense effects. Plant Mol. Biol. 14: 457-466

Vernon, L., (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal. Chem. 32: 1144-1150

von Wettstein, D., Gough, S., and Kannangara, CG., (1995) Chlorophyll Biosynthesis. The Plant Cell 7: 1039-1057

von Wettstein, D., (1958) The formation of plastid structure. In The Photochemical Apparatus, Its Structure and Function. Brookhaven Symposium Biology II. Upton, NY: Brookhaven National Laboratory. pp. 138-159

Wang, K. Herrera-Estrella, L., Van Montagu, M., and Zambryski, P., (1984) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38: 455-462

Watson, B., Currier, T., Gordon, M., Chilton, M., and Nester, E., (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 123: 255-264

Westhoff, P., and Hermann, R., (1988) Complex RNA maturation in chloroplasts. Eur. J. Biochem. 171: 551-567

White, M., and Green, B., (1987a) Antibodies to the Photosystem I chlorophyll a+b antenna cross-react with polypeptides of CP29 and LHCII. Eur. J. Biochem. 165: 545-551

White, M., and Green, B., (1987b) Polypeptides belonging to each of the three major chlorophyll a+b protein complexes are present in a chlorophyll-b-less barley mutant. Eur. J. Biochem. 165: 531-535

White, M., and Green, B., (1988) Intermittent-light chloroplasts are not developmentally equivalent to *chlorina* f2 chloroplasts in barley. Photosynthesis Research 15: 195-203

White, M., et al., (1992) Expression of the chlorophyll a/b protein multigene family in pea (*Pisum sativum* L): Evidence for distinct developmental responses. Planta 186: 190-198

White, M., Kaufman, L., Horwitz, B., Briggs, W., and Thompson, W., (1995) Individual members of the cab gene family differ widely in fluence response. Plant Physiol. 107: 161-165

Woolhouse, H., (1987) Regulation of senescence in the chloroplast. In: Thomson, W. W., E.A. Nothnagel, and R.C. Huffaker, eds., Plant Senescence: Its Biochemistry and Physiology, The Amer. Soc. Plant Physiol., pp. 132-145

Yanisch-Perron, C., Vieira, J., and Messing, J., (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequence of M13mp18 and vectors. Gene 33: 103-110

Zambryski, P., et al., (1982) Tumor induction by *Agrobacterium tumefaciens*: Analysis of the boundaries of T-DNA. J. Mol. App. Genet. 1: 361-370

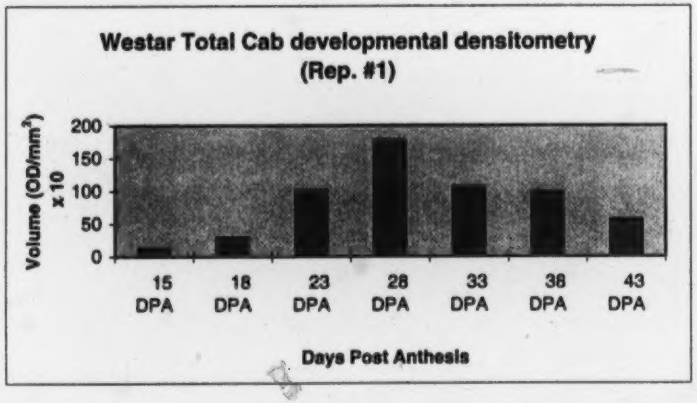
Ziegler, R., and Schanderl, S., (1969) Chlorophyll degradation and the kinetics of dephytylated derivatives in a mutant of *Chlorella*. Photosynthetica 3: 45-54

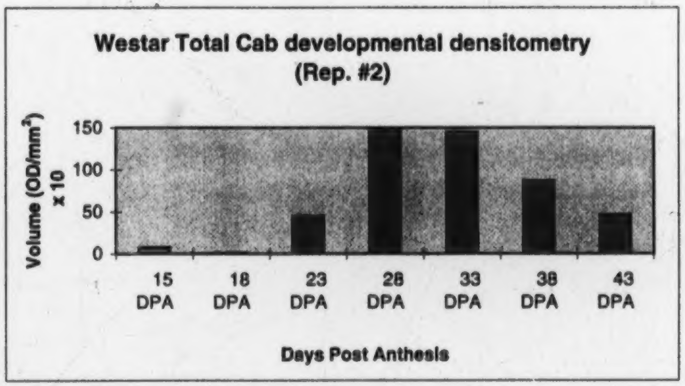
9: APPENDICES

9.I: APPENDIX A - DEVELOPMENTAL DENSITOMETRY OF CAB ACCUMULATION IN SEED OF *BRASSICA NAPUS*

Western blot analysis was performed to examine Cab protein accumulation in developing seed of Westar, Control and T2 seed of DII and DIII. Densitometric analysis of the western blots revealed maximum Cab accumulation at 28 DPA in all examined plant lines (Fig. A-1 through Fig. A-4).

FIG. A-1: Densitometry analysis of western blots showing Cab protein accumulation in seed of *Brassica napus* cv. Westar during development. Total protein samples, representing each of the 7 examined DPA, were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.


Westar Total Cab developmental densitometry (Rep. #1)


Westar Total Cab developmental densitometry (Rep. #2)

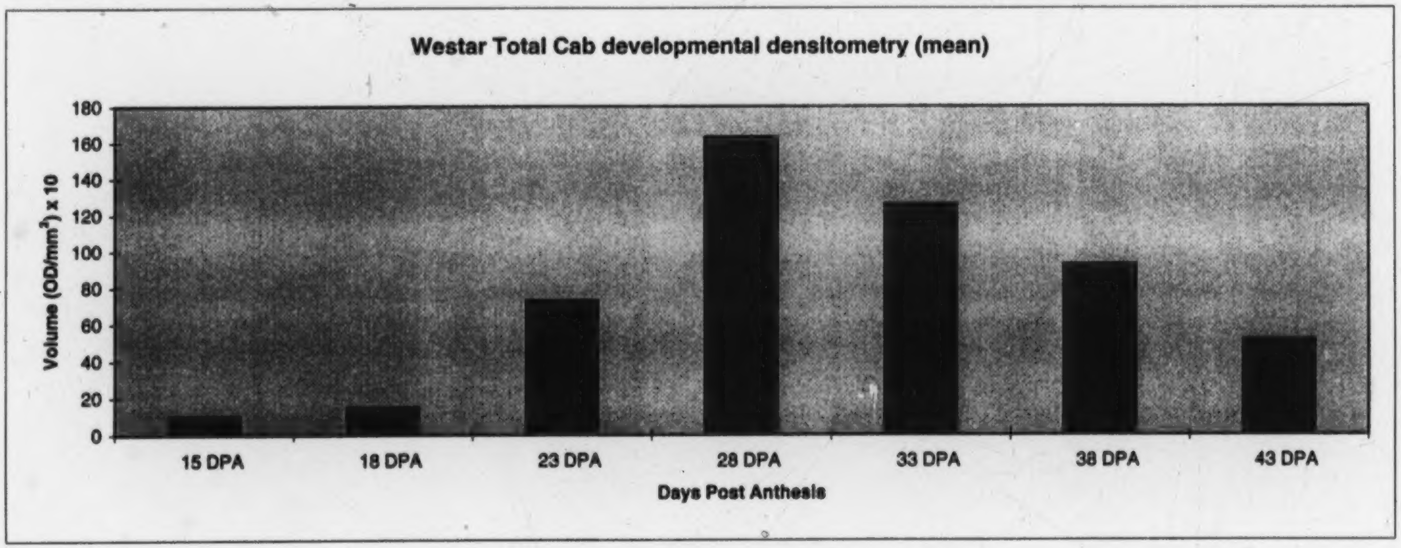
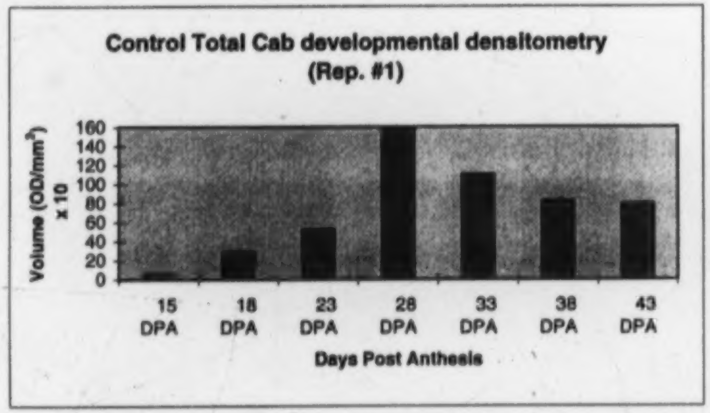
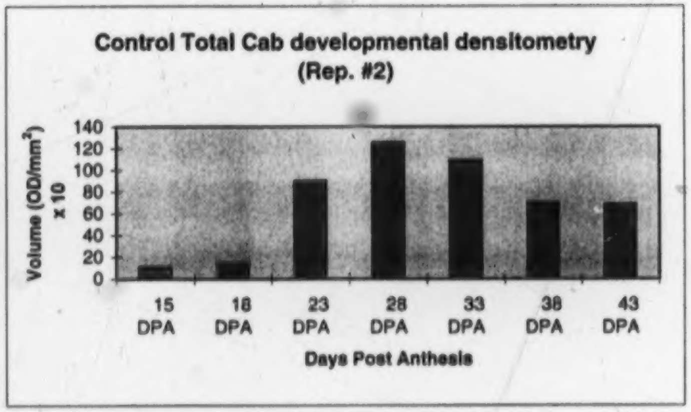

Westar Total Cab developmental densitometry (mean)

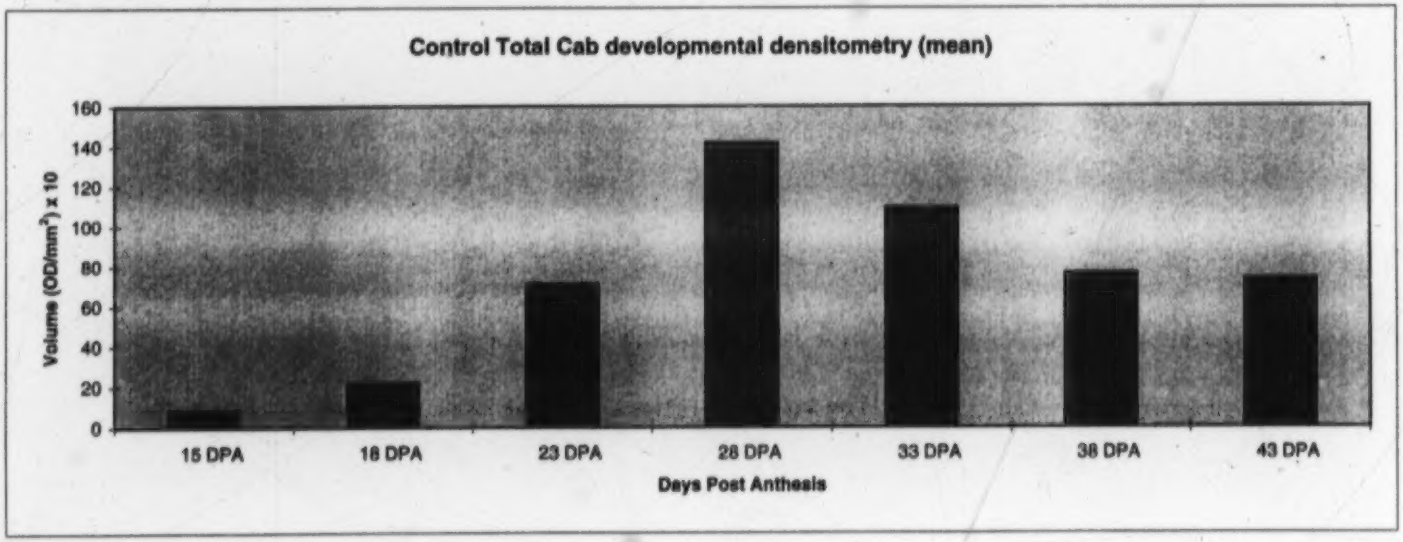
FIG. A-2: Densitometry analysis of western blots showing Cab protein accumulation in seed of Control plants during development. Total protein samples, representing each of the 7 examined DPA, were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.



Control Total Cab developmental densitometry (Rep. #1)

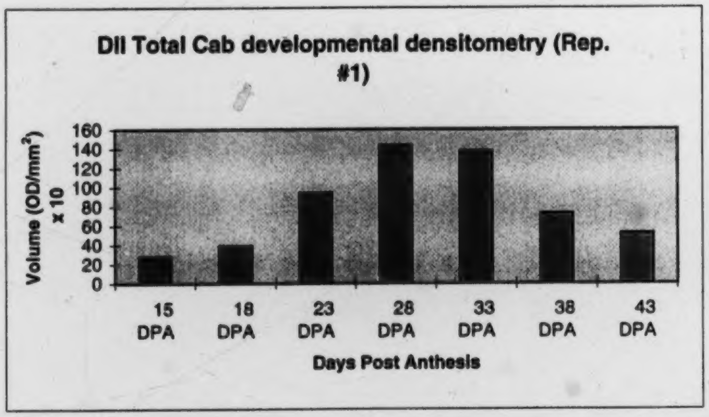


Control Total Cab developmental densitometry (Rep. #2)

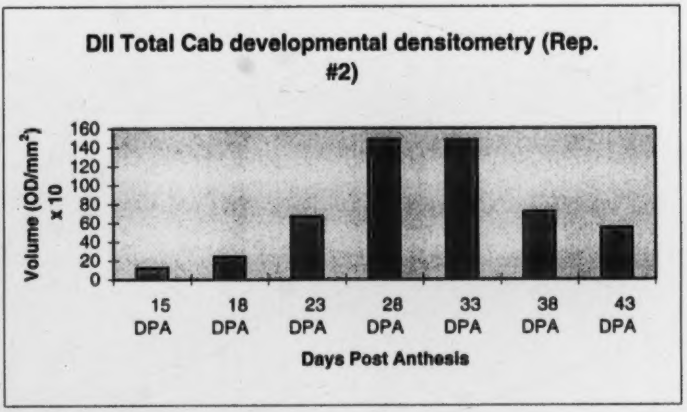


Control Total Cab developmental densitometry (mean)

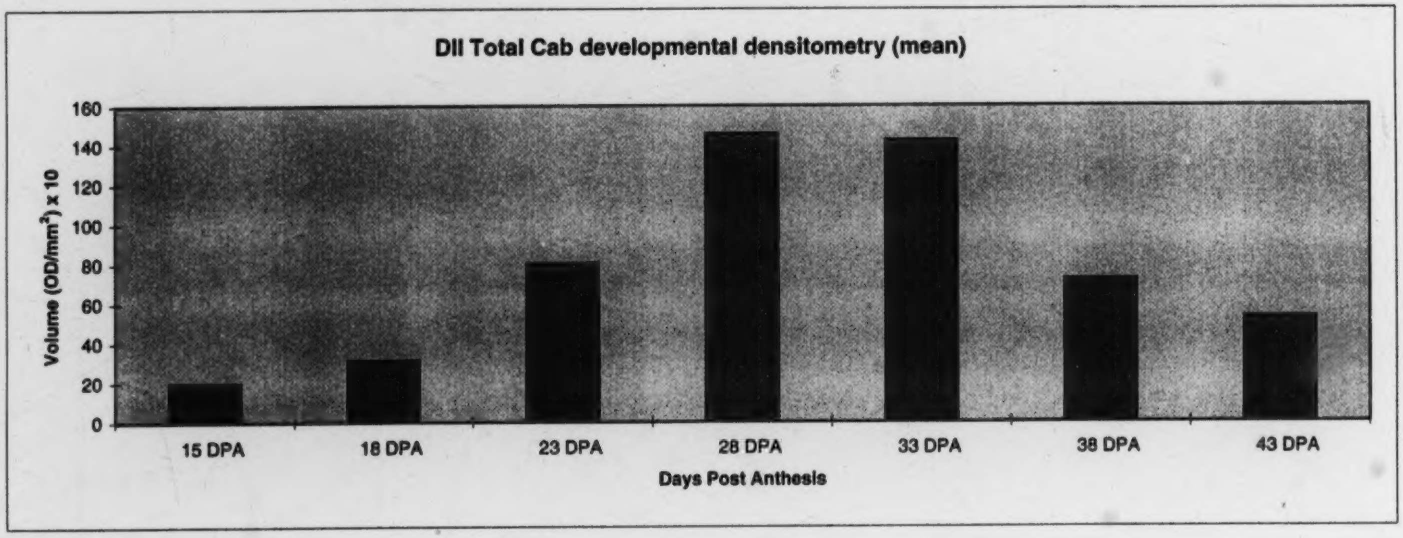
FIG. A-3: Densitometry analysis of western blots showing Cab protein accumulation in T2 seed of the *Brassica napus* antisense cab transgenic, DII, during development. Total protein samples, representing each of the 7 examined DPA, were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2^{o} antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.



DII Total Cab developmental densitometry (Rep. #1)

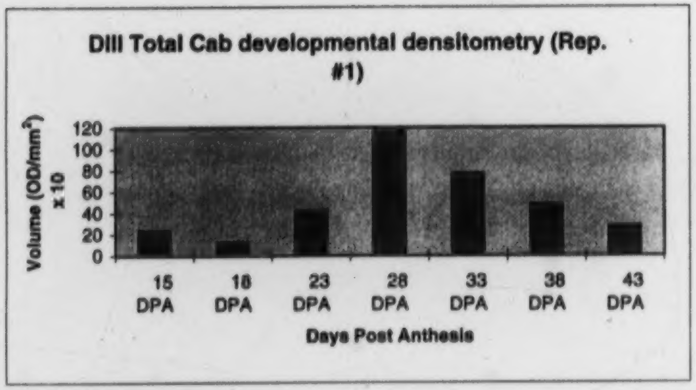


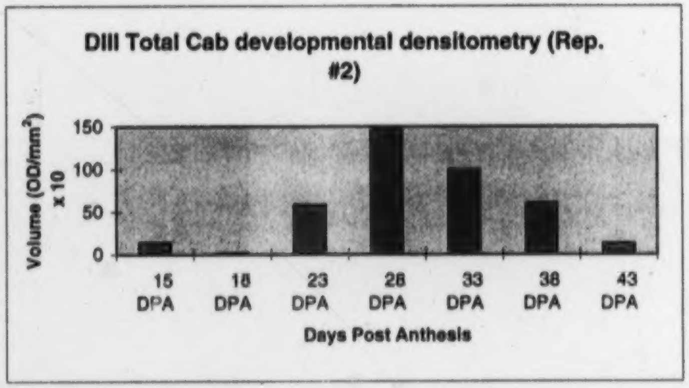
DII Total Cab developmental densitometry (Rep. #2)

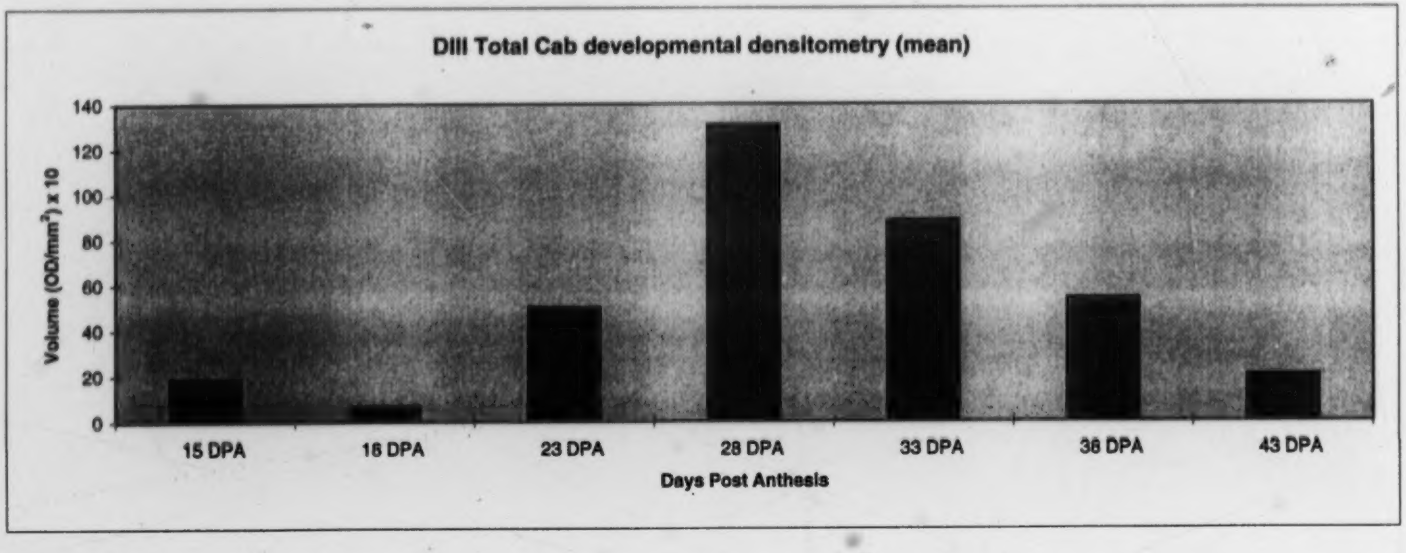


DII Total Cab developmental densitometry (mean)

FIG. A-4: Densitometry analysis of western blots showing Cab protein accumulation in T2 seed of the *Brassica napus* antisense cab transgenic, DIII, during development. Total protein samples, representing each of the 7 examined DPA, were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.


DIII Total Cab developmental densitometry (Rep. #1)

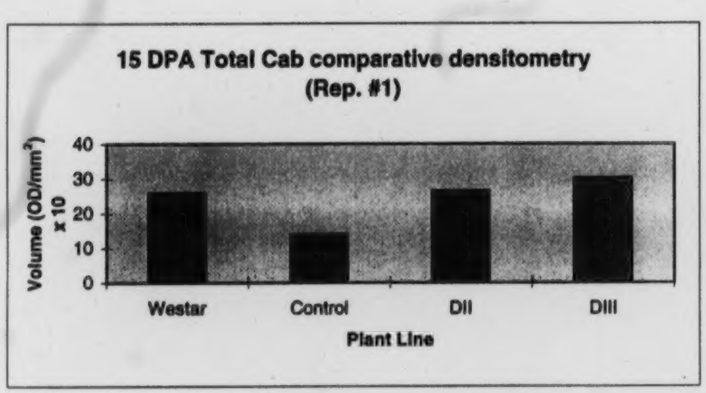

DIII Total Cab developmental densitometry (Rep. #2)


DIII Total Cab developmental densitometry (mean)

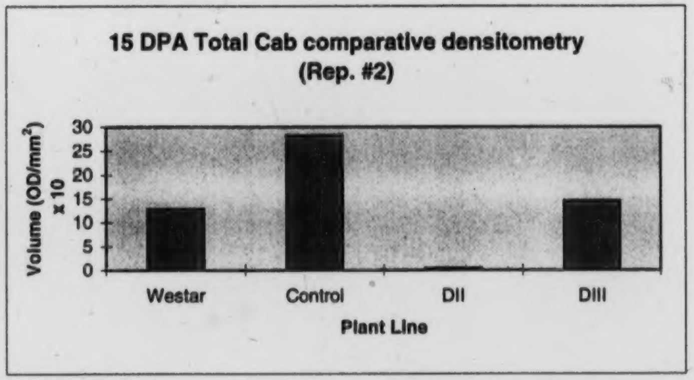
9.II: APPENDIX B - COMPARATIVE DENSITOMETRY OF CAB ACCUMULATION IN SEED OF *BRASSICA NAPUS*

Western blots were probed with α-CPIa to detect Cab accumulation in seed of Westar, Control and T2 seed of DII and DIII at various DPA.

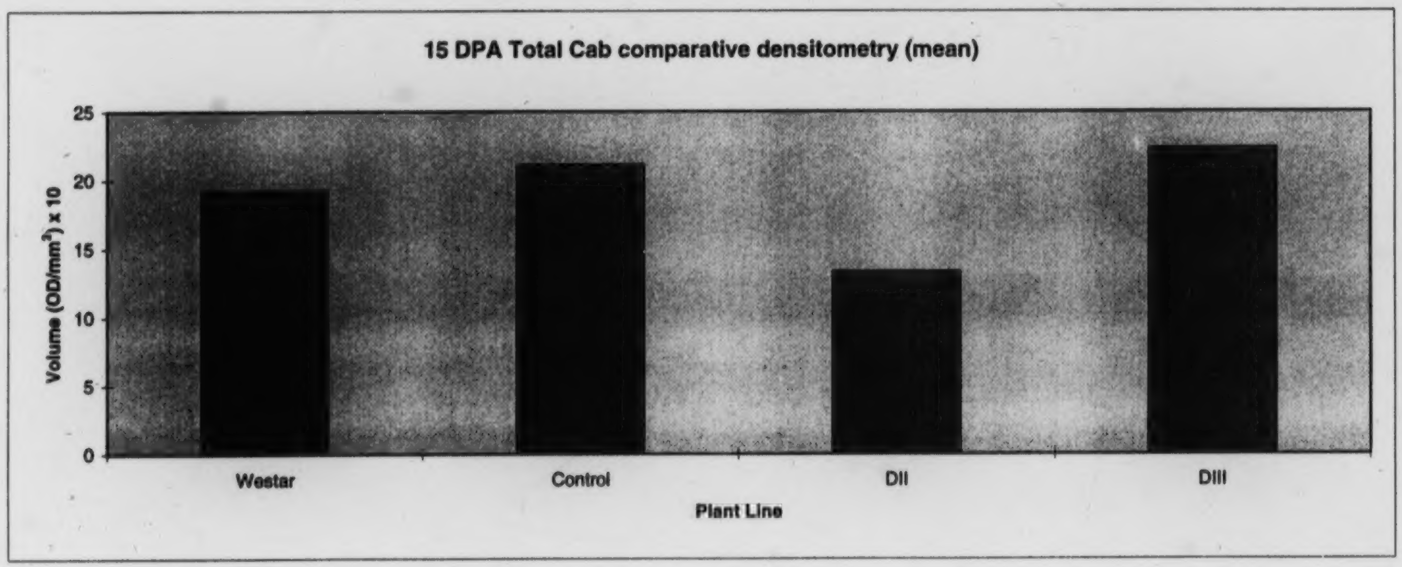
FIG. B-1: Densitometry analysis of western blots showing Cab protein accumulation in Westar and Control seed and T2 seed of DII and DIII at 15 DPA. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.



15 DPA Total Cab comparative densitometry (Rep. #1)



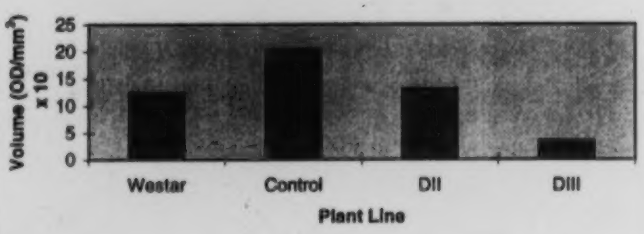
15 DPA Total Cab comparative densitometry (Rep. #2)



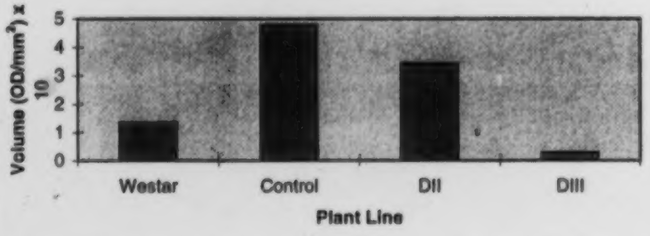
15 DPA Total Cab comparative densitometry (mean)

FIG. B-2: Densitometry analysis of western blots showing Cab protein accumulation in seed of Westar and Control,and T2 seed of DII and DIII at 18 DPA. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.

18 DPA LHCIIa comparative densitometry (Rep. #1)



18 DPA LHCI comparative densitometry (Rep. #1)



18 DPA Total Cab comparative densitometry (Rep. #1)

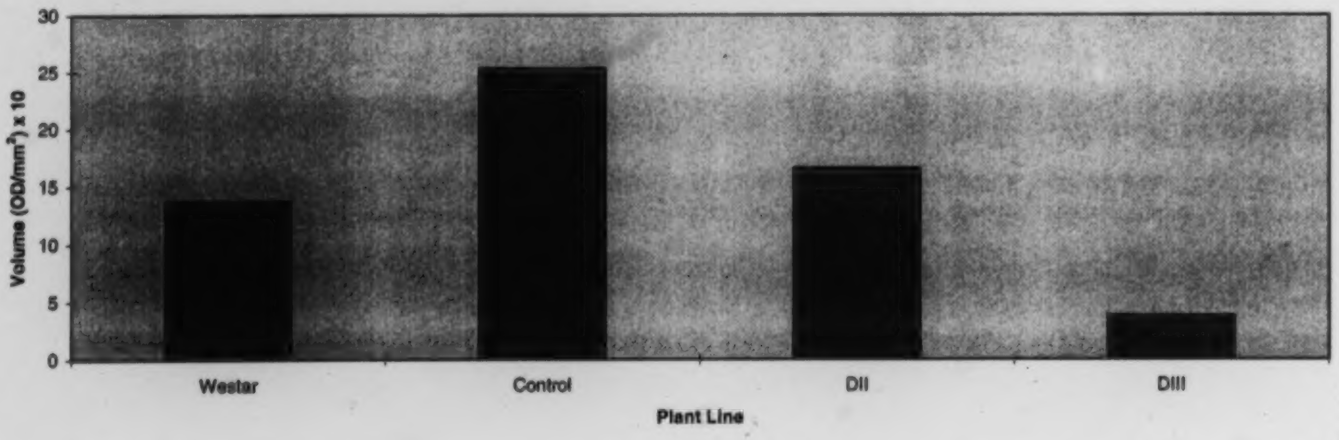
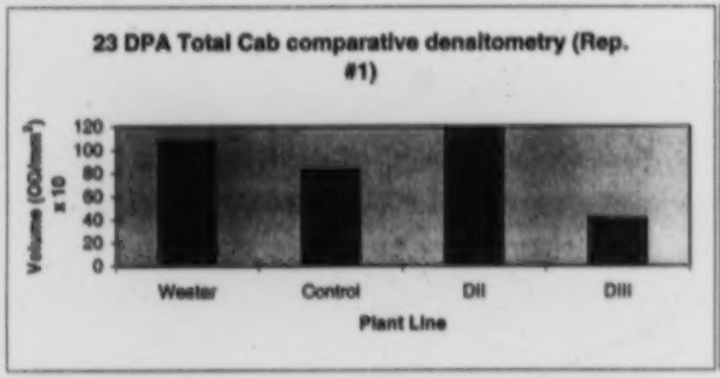
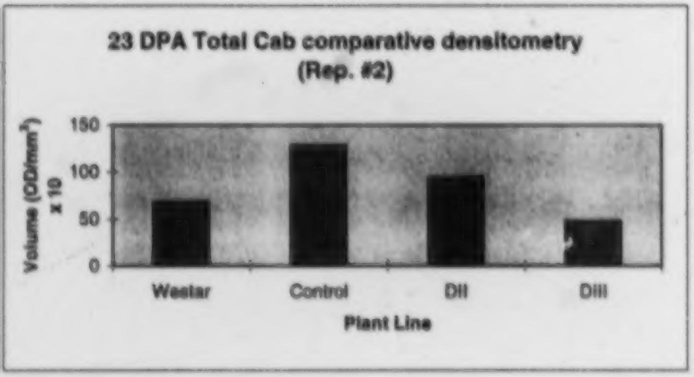


FIG. B-3: Densitometry analysis of western blots showing Cab protein accumulation in seed of Westar and Control, and T2 seed of DII and DIII at 23 DPA. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.


23 DPA Total Cab comparative densitometry (Rep. #1)


23 DPA Total Cab comparative densitometry (Rep. #2)

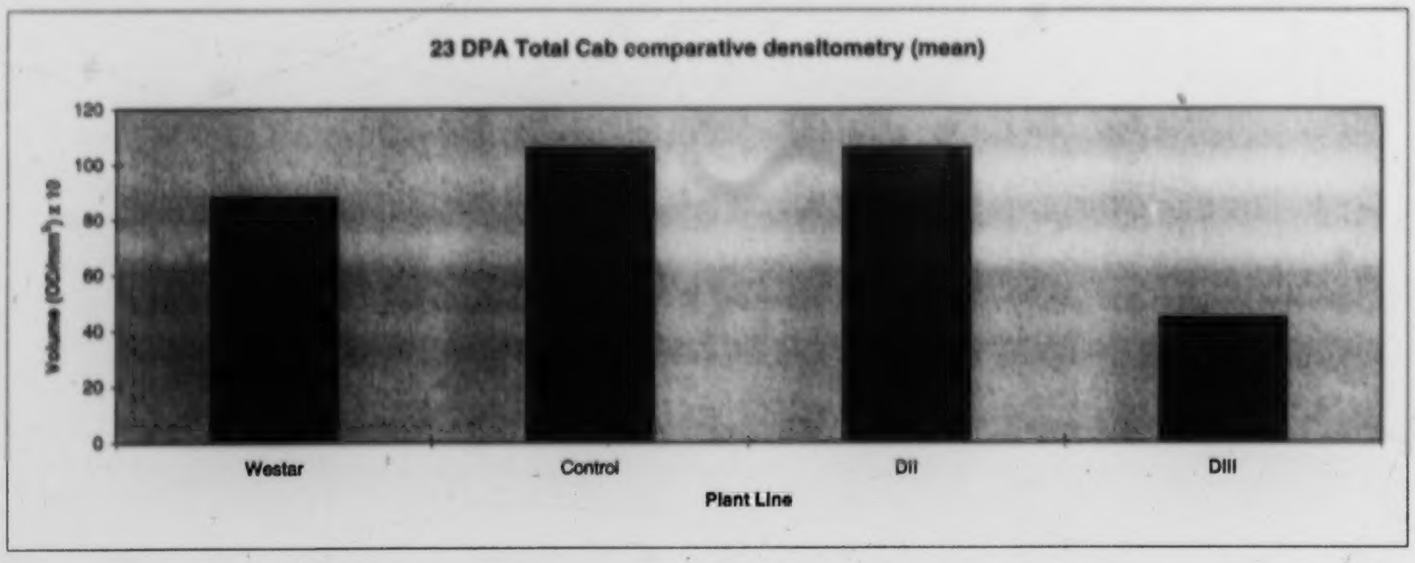
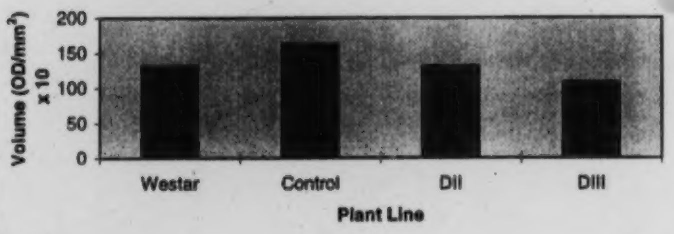
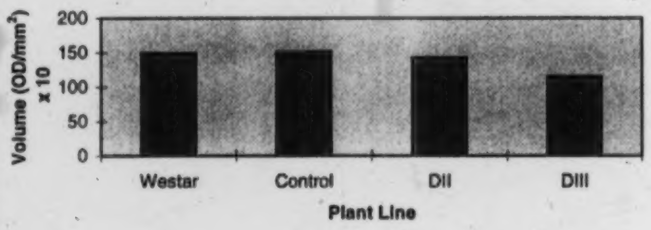

23 DPA Total Cab comparative densitometry (mean)

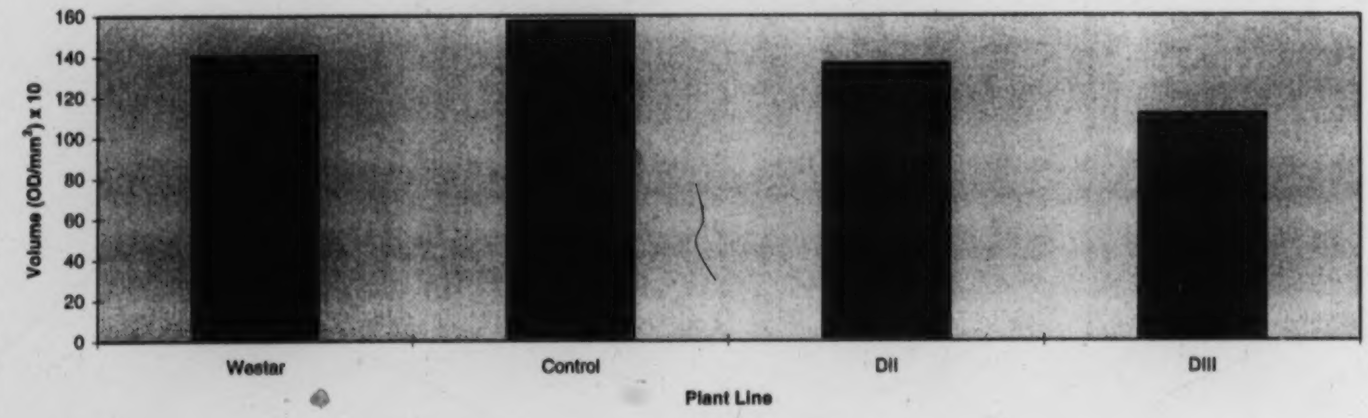
FIG. B-4: Densitometry analysis of western blots showing Cab protein accumulation in seed of Westar and Control, and T2 seed of DII and DIII at 28 DPA. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.



28 DPA Total Cab comparative densitometry (Rep. #1)

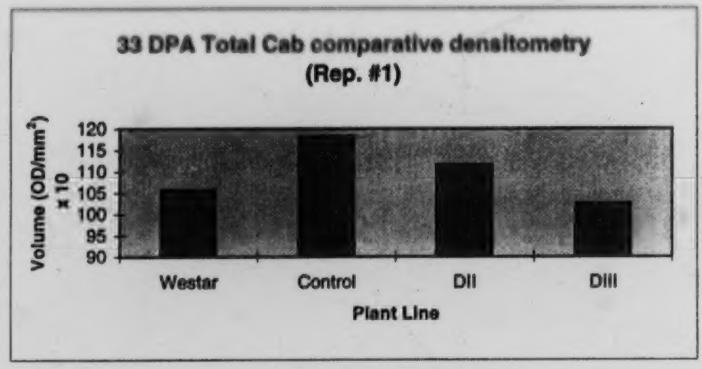


28 DPA Total Cab comparative densitometry (Rep. #2)

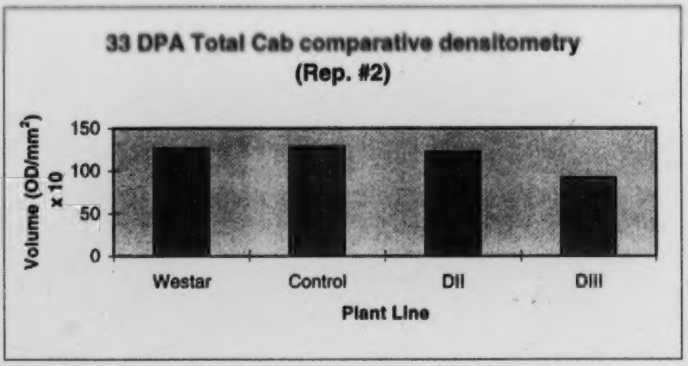


28 DPA Total Cab comparative densitometry (mean)

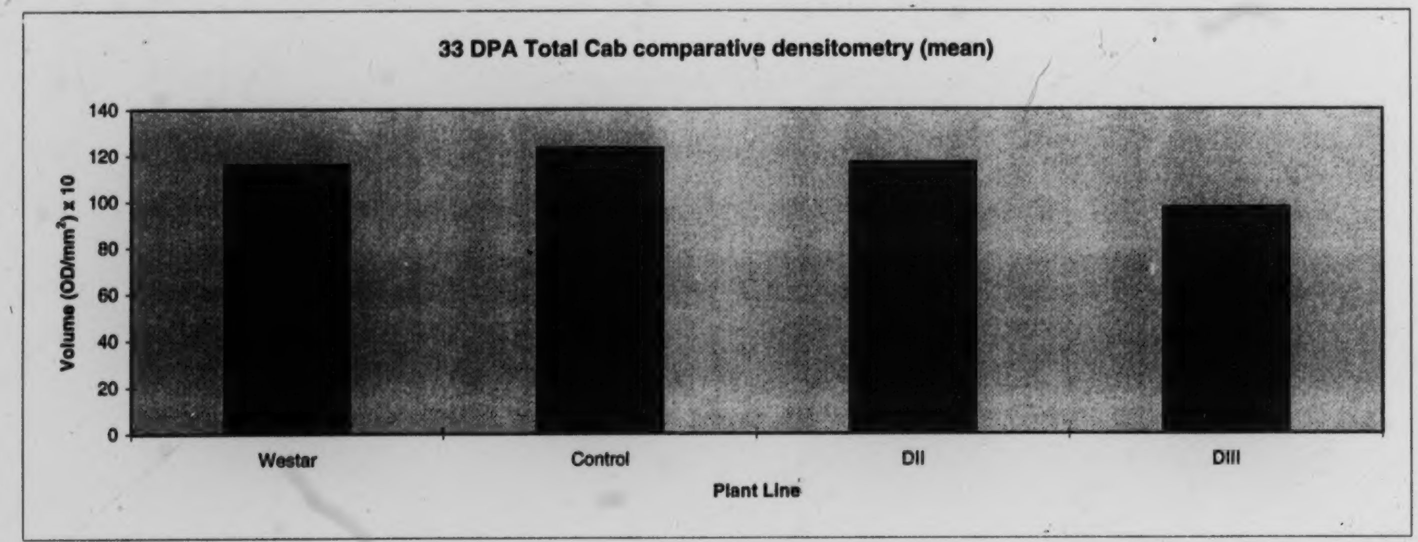
FIG. B-5: Densitometry analysis of western blots showing Cab protein accumulation in seed of Westar and Control, and T2 seed of DII and DIII at 33 DPA. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.



33 DPA Total Cab comparative densitometry (Rep. #1)

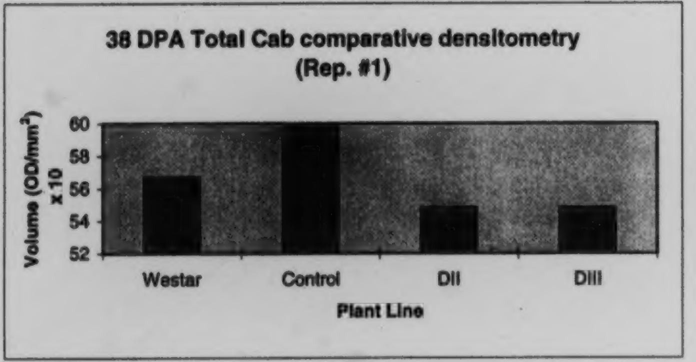


33 DPA Total Cab comparative densitometry (Rep. #2)

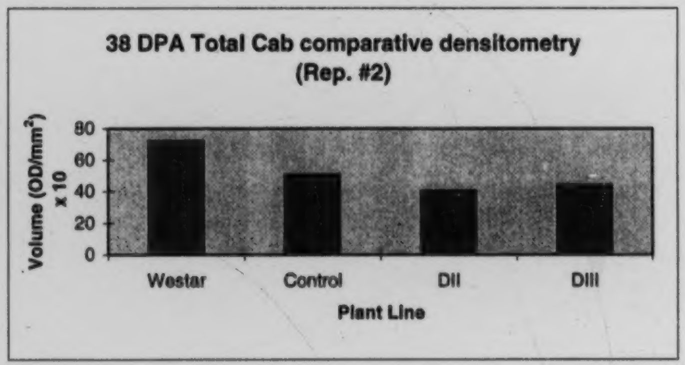


33 DPA Total Cab comparative densitometry (mean)

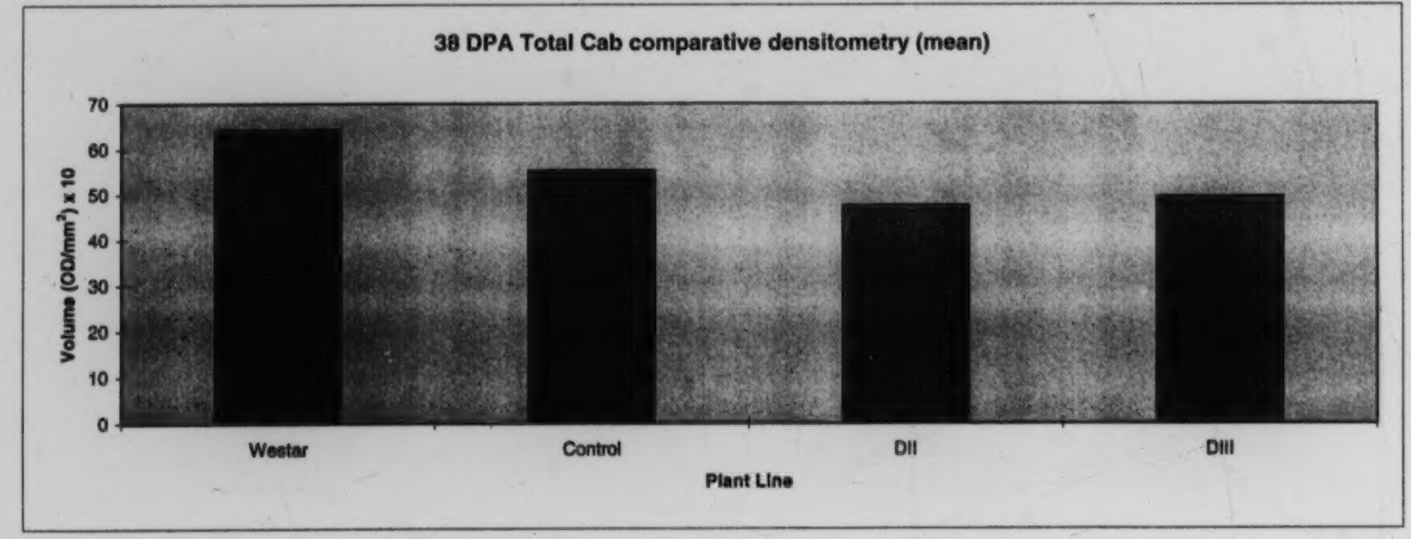
FIG. B-6: Densitometry analysis of western blots showing Cab protein accumulation in seed of Westar and Control, and T2 seed of DII and DIII at 38 DPA. Total protein samples were loaded onto 12.5% SDS-PAGE gels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.



38 DPA Total Cab comparative densitometry (Rep. #1)

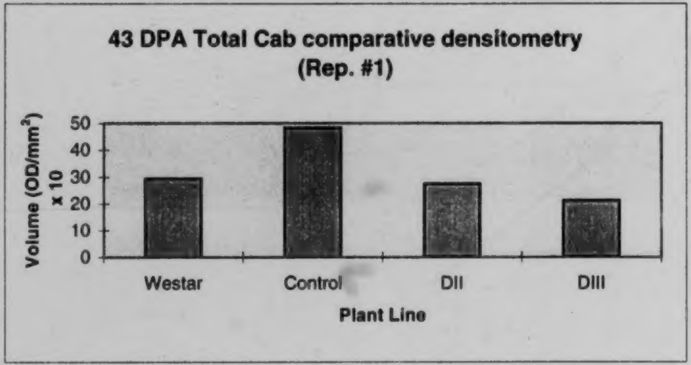


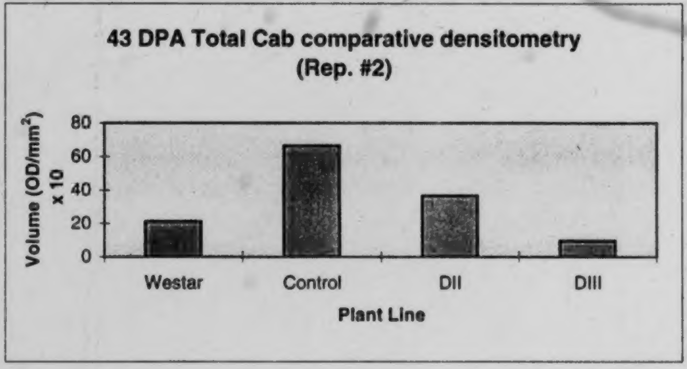
38 DPA Total Cab comparative densitometry (Rep. #2)

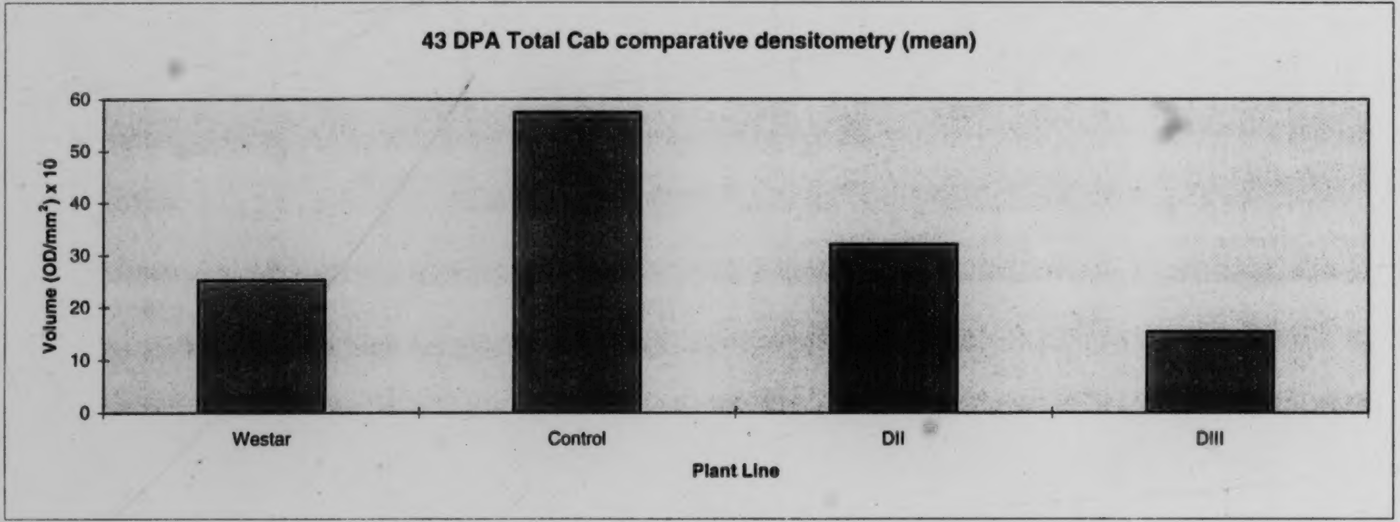


38 DPA Total Cab comparative densitometry (mean)

FIG. B-7: Densitometry analysis of western blots showing Cab protein accumulation in seed of Westar and Control, and T2 seed of DII and DIII at 43 DPA. Total protein samples were loaded onto 12.5% SDS-PAGEgels on a per seed basis (0.5 seed/lane), separated, transferred to nitrocellulose, probed with α-CPIa and detected with 2° antibody conjugated to alkaline phosphatase. Once western blots were developed, the image was scanned and the bands analyzed using densitometry techniques. The calculated volumes were representative of the quantity of Cab polypeptide in each western band. Total Cab was calculated from component bands. The upper left and right graphs represent analysis of 2 replicate western blots. The lower graph represents the means of the data from the 2 replicate graphs.


43 DPA Total Cab comparative densitometry
(Rep. #1)
Volume (OD/mm²) x 10
50
40
30
20
10
0
Westar
Control
DII
DIII
Plant Line


43 DPA Total Cab comparative densitometry
(Rep. #2)
Volume (OD/mm²) x 10
80
60
40
20
0
Westar
Control
DII
DIII
Plant Line


43 DPA Total Cab comparative densitometry (mean)
Volume (OD/mm²) x 10
60
50
40
30
20
10
0
Westar
Control
DII
DIII
Plant Line